

1 Chimeric binding peptide library screening method

2

3 The present invention relates generally to methods for
4 screening nucleotide libraries for sequences that
5 encode peptides of interest.

6

7 Isolating an unknown gene which encodes a desired
8 peptide from a recombinant DNA library can be a
9 difficult task. The use of hybridisation probes may
10 facilitate the process, but their use is generally
11 dependent on knowing at least a portion of the sequence
12 of the gene which encodes the protein. When the
13 sequence is not known, DNA libraries can be expressed
14 in an expression vector, and antibodies have been used
15 to screen for plaques or colonies displaying the
16 desired protein antigen. This procedure has been useful
17 in screening small libraries, but rarely occurring
18 sequences which are represented in less than about 1 in
19 10^5 clones (as is the case with rarely occurring cDNA
20 molecules or synthetic peptides) can be easily missed,
21 making screening libraries larger than 10^6 clones at
22 best laborious and difficult. Methods designed to
23 address the isolation of rarely occurring sequences by
24 screening libraries of 10^6 clones have been developed
25 and include phage display methods and LacI fusion phage
26 display, discussed in more detail below.

27

28 Phage display methods. Members of DNA libraries which
29 are fused to the N-terminal end of filamentous
30 bacteriophage pIII and pVIII coat proteins have been
31 expressed from an expression vector resulting in the

1 display of foreign peptides on the surface of the phage
2 particle with the DNA encoding the fusion protein
3 packaged in the phage particle (Smith G. P., 1985,
4 Science 228: 1315-1317). The expression vector can be
5 the bacteriophage genome itself, or a phagemid vector,
6 into which a bacteriophage coat protein has been
7 cloned. In the latter case, the host bacterium,
8 containing the phagemid vector, must be co-infected
9 with autonomously replicating bacteriophage, termed
10 helper phage, to provide the full complement of
11 proteins necessary to produce mature phage particles.
12 The helper phage normally has a genetic defect in the
13 origin of replication which results in the preferential
14 packaging of the phagemid genome. Expression of the
15 fusion protein following helper phage infection, allows
16 incorporation of both fusion protein and wild type coat
17 protein into the phage particle during assembly.
18 Libraries of fusion proteins incorporated into phage,
19 can then be selected for binding members against
20 targets of interest (ligands). Bound phage can then be
21 allowed to reinfect *Escherichia coli* (*E. coli*) bacteria
22 and then amplified and the selection repeated,
23 resulting in the enrichment of binding members
24 (Parmley, S. F., & Smith, G. P. 1988., Gene 73: 305-
25 318; Barrett R. W. et al., 1992, Analytical
26 Biochemistry 204: 357-364 Williamson et al., Proc.
27 Natl. Acad. Sci. USA, 90: 4141-4145; Marks et al.,
28 1991, J. Mol. Biol. 222: 581-597).
29
30 Several publications describe this method. For example,
31 US Patent No 5,403,484 describes production of a

1 chimeric protein formed from the viral coat protein and
2 the peptide of interest. In this method at least a
3 functional portion of a viral coat protein is required
4 to cause display of the chimeric protein or a processed
5 form thereof on the outer surface of the virus. In
6 addition, US Patent No 5,571,698 describes a method for
7 obtaining a nucleic acid encoding a binding protein, a
8 key component of which comprises preparing a population
9 of amplifiable genetic packages which have a
10 genetically determined outer surface protein, to cause
11 the display of the potential binding domain on the
12 outer surface of the genetic package. The genetic
13 packages are selected from the group consisting of
14 cells, spores and viruses. For example when the
15 genetic package is a bacterial cell, the outer surface
16 transport signal is derived from a bacterial outer
17 surface protein, and when the genetic package is a
18 filamentous bacteriophage, the outer surface transport
19 signal is provided by the gene pIII (minor coat
20 protein) or pVIII (major coat protein) of the
21 filamentous phage.

22

23 WO-A-92/01047 and WO-A-92/20791 describe methods for
24 producing multimeric specific binding pairs, by
25 expressing a first polypeptide chain fused to a viral
26 coat protein, such as the gene pIII protein, of a
27 secreted replicable genetic display package (RGDP)
28 which displays a polypeptide at the surface of the
29 package, and expressing a second polypeptide chain of
30 the multimer, and allowing the two chains to come
31 together as part of the RGDP.

1
2 LacI fusion plasmid display. This method is based on
3 the DNA binding ability of the lac repressor. Libraries
4 of random peptides are fused to the lacI repressor
5 protein, normally to the C-terminal end, through
6 expression from a plasmid vector carrying the fusion
7 gene. Linkage of the LacI-peptide fusion to its
8 encoding DNA occurs via the lacO sequences on the
9 plasmid, forming a stable peptide-LacI-peptide complex.
10 These complexes are released from their host bacteria
11 by cell lysis, and peptides of interest isolated by
12 affinity purification on an immobilised target. The
13 plasmids thus isolated can then be reintroduced into *E.*
14 *coli* by electroporation to amplify the selected
15 population for additional rounds of screening (Cull, M.
16 G. et al. 1992. Proc. Natl. Acad. Sci. U.S.A. 89:1865-
17 1869).

18
19 US Patent No 5498530 describes a method for
20 constructing a library of random peptides fused to a
21 DNA binding protein in appropriate host cells and
22 culturing the host cells under conditions suitable for
23 expression of the fusion proteins intra-cellularly, in
24 the cytoplasm of the host cells. This method also
25 teaches that the random peptide is located at the
26 carboxy terminus of the fusion protein and that the
27 fusion protein-DNA complex is released from the host
28 cell by cell lysis. No method is described for the
29 protection of the DNA from degradation once released
30 from the lysed cell. Several DNA binding proteins are
31 claimed but no examples are shown except lacI.

1
2 There remains a need for methods of constructing
3 peptide libraries in addition to the methods described
4 above. For instance, the above methods do not permit
5 production of secreted peptides with a free carboxy
6 terminus. The present invention describes an
7 alternative method for isolating peptides of interest
8 from libraries and has significant advantages over the
9 prior art methods.

10
11 In general terms, the present invention provides a
12 method for screening a nucleotide library (usually a
13 DNA library) for a nucleotide sequence which encodes a
14 target peptide of interest. The method involves
15 physically linking each peptide to a polynucleotide
16 including the specific nucleotide sequence encoding
17 that peptide. Linkage of a peptide to its encoding
18 nucleotide sequence is achieved via linkage of the
19 peptide to a nucleotide binding domain. A bifunctional
20 chimeric protein with a nucleotide binding domain and a
21 library member or target peptide (preferably with a
22 function of interest) is thus obtained. The peptide of
23 interest is bound to the polynucleotide encoding that
24 peptide via the nucleotide binding domain of the
25 chimeric protein.

26
27 The polynucleotide-chimeric protein complex is then
28 incorporated within a peptide display carrier package
29 (PDCP), protecting the polynucleotide from subsequent
30 degradation, while displaying the target peptide

1 portion on the outer surface of the peptide display
2 carrier package (PDCP).

3
4 Thus, in one aspect, the present invention provides a
5 peptide display carrier package (PDCP), said package
6 comprising a polynucleotide-chimeric protein complex
7 wherein the chimeric protein has a nucleotide binding
8 portion and a target peptide portion, wherein said
9 polynucleotide comprises a nucleotide sequence motif
10 which is specifically bound by said nucleotide binding
11 portion, and wherein at least the chimeric protein
12 encoding portion of the polynucleotide not bound by the
13 nucleotide binding portion of the chimeric protein is
14 protected.

15
16 In one embodiment the polynucleotide is protected by a
17 protein which binds non-specifically to naked
18 polynucleotide. Examples include viral coat proteins,
19 many of which are well-known in the art. Where the
20 chosen viral coat protein requires an initiation
21 sequence to commence general binding to the
22 polynucleotide, this will be provided on the
23 polynucleotide at appropriate location(s). A preferred
24 coat protein is coat protein from a bacteriophage,
25 especially M13.

26
27 Generally, the nucleic binding portion of the chimeric
28 protein is selected for its specificity for the
29 nucleotide sequence motif present in the recombinant
30 polynucleotide encoding the chimeric protein.

31

1 Optionally, the nucleotide sequence motif may be an
2 integral part of the protein encoding region of the
3 polynucleotide. Alternatively, and more usually, the
4 motif may be present in a non-coding region of the
5 polynucleotide. For the purposes of this invention,
6 all that is required is for the motif to be located on
7 the polynucleotide such that the nucleotide binding
8 portion of the chimeric protein is able to recognise
9 and bind to it. Desirably the polynucleotide-chimeric
10 protein complex has a dissociation constant of at least
11 one hour.

12

13 Optionally, the recombinant polynucleotide may comprise
14 two or more nucleotide sequence motifs, each of which
15 will be bound by a chimeric protein molecule.
16 Preferably, the motifs are positioned along the length
17 of the polynucleotide to avoid steric hindrance between
18 the bound chimeric proteins.

19

20 Preferably, the nucleotide sequence motif is not
21 affected by the presence of additional nucleotide
22 sequence (e.g. encoding sequence) at its 5' and/or 3'
23 ends. Thus the chimeric fusion protein may include a
24 target peptide portion at its N terminal end, at its C
25 terminal end or may include two target peptide portions
26 (which may be the same or different) at each end of the
27 nucleotide binding portion, ie at both the N and C
28 terminal ends of the chimeric protein. For example one
29 target peptide may be an antibody of known specificity
30 and the other peptide may be a peptide of potential
31 interest.

1
2 Desirably the target peptide portion of the chimeric
3 protein is displayed externally on the peptide display
4 carrier package, and is thus available for detection,
5 reaction and/or binding.

6
7 In more detail the PDCP may be composed two distinct
8 elements:

- 9 a. A polynucleotide-chimeric protein complex. This
10 links the displayed target peptide portion to the
11 polynucleotide encoding that peptide portion
12 through a specific polynucleotide binding portion.
13 The nucleotide sequence encoding the chimeric
14 protein, and the specific nucleotide sequence
15 motif recognised by the nucleotide binding portion
16 of the chimeric protein must be present on a
17 segment of polynucleotide which can be
18 incorporated into the PDCP; and
19 b. A protective coat. This may be supplied by a
20 replicable carrier or helper package capable of
21 independent existence. Alternatively, a coat
22 protein could be encoded by the recombinant
23 polynucleotide of the invention. The protective
24 coat for the polynucleotide-chimeric protein
25 complex may be composed of a biological material
26 such as protein or lipid, but the protective coat
27 is not required for linking the target peptide to
28 the polynucleotide encoding that peptide. The
29 protective coat must allow the display of the
30 target peptide portion of the chimeric protein on
31 its outer surface. The carrier or helper package

1 may also provide the mechanism for releasing the
2 intact PDCP from host cells when so required. By
3 way of example, when a bacteriophage is the
4 replicable carrier package, a protein coat of the
5 bacteriophage surrounds the polynucleotide-
6 chimeric protein complex to form the PDCP, which
7 is then extruded from the host bacterial cell.

8
9 The invention described herein demonstrates that
10 peptides fused to a nucleotide binding domain can be
11 displayed externally, even through a bacteriophage
12 carrier package protein coat, while still bound to the
13 polynucleotide encoding the displayed peptide.

14
15 The present invention also provides a recombinant
16 polynucleotide comprising a nucleotide sequence
17 encoding a chimeric protein having a nucleotide binding
18 portion operably linked to a target peptide portion,
19 wherein said polynucleotide includes a specific
20 nucleotide sequence motif which is bound by the
21 nucleotide binding portion of said chimeric protein and
22 further encoding a non-sequence-specific nucleotide
23 binding protein.

24
25 Desirably, the recombinant polynucleotide is a
26 recombinant expression system, able to express the
27 chimeric protein when placed in a suitable environment,
28 for example a compatible host cell. After its
29 expression, the chimeric protein binds to the specific
30 nucleotide sequence (motif) present in the

1 polynucleotide comprising the nucleotide sequence
2 encoding the chimeric protein.

3
4 Optionally there may be a linker sequence located
5 between the nucleotide sequence encoding the nucleotide
6 binding portion and the polynucleotide inserted into
7 the restriction enzyme site of the construct.

8
9 Desirably the nucleotide binding portion is a DNA
10 binding domain of an [oestrogen] estrogen or
11 progesterone receptor, or a functional equivalent
12 thereof. Examples of sequences encoding such
13 nucleotide binding portions are set out in SEQ ID Nos
14 11 and 13.

15
16 The term "expression system" is used herein to refer to
17 a genetic sequence which includes a protein-encoding
18 region and is operably linked to all of the genetic
19 signals necessary to achieve expression of that region.
20 Optionally, the expression system may also include
21 regulatory elements, such as a promoter or enhancer to
22 increase transcription and/or translation of the
23 protein encoding region or to provide control over
24 expression. The regulatory elements may be located
25 upstream or downstream of the protein encoding region
26 or within the protein encoding region itself. Where
27 two or more distinct protein encoding regions are
28 present these may use common regulatory element(s) or
29 have separate regulatory element(s).
30

1 Generally, the recombinant polynucleotide described
2 above will be DNA. Where the expression system is
3 based upon an M13 vector, usually the polynucleotide
4 binding portion of the expressed chimeric portion will
5 be single-stranded DNA. However, other vector systems
6 may be used and the nucleotide binding portion may be
7 selected to bind preferentially to double-stranded DNA
8 or to double or single-stranded RNA, as convenient.

9
10 Additionally the present invention provides a vector
11 containing such a recombinant expression system and
12 host cells transformed with such a recombinant
13 expression system (optionally in the form of a vector).

14
15 Whilst the recombinant polynucleotide described above
16 forms an important part of the present invention, we
17 are also concerned with the ability to screen large
18 (e.g. of at least 10^5 members, for example 10^6 or even
19 10^7 members) libraries of genetic material. One of the
20 prime considerations therefore is the provision of a
21 recombinant genetic construct into which each member of
22 said library can individually be incorporated to form
23 the recombinant polynucleotide described above and to
24 express the chimeric protein thereby encoded (the
25 target peptide of which is encoded by the nucleotide
26 library member incorporated into the construct).

27
28 Thus viewed in a further aspect the present invention
29 provides a genetic construct or set of genetic
30 constructs comprising a polynucleotide having a
31 sequence which includes:

- 1
2 i) a sequence encoding a nucleotide binding portion
3 able to recognise and bind to a specific sequence
4 motif;
5 ii) the sequence motif recognised and bound by the
6 nucleotide binding portion encoded by (i);
7 iii) a restriction enzyme site which permits insertion
8 of a polynucleotide, said site being designed to
9 operably link said polynucleotide to the sequence
10 encoding the nucleotide binding portion so that
11 expression of the operably linked polynucleotide
12 sequences yields a chimeric protein; and
13 iv) a sequence encoding a nucleotide binding protein
14 which binds non-specifically to naked
15 polynucleotide.

16
17 Optionally there may be a linker sequence located
18 between the nucleotide sequence encoding the nucleotide
19 binding portion and the sequence of the polynucleotide
20 from the library inserted into the restriction enzyme
21 site of the construct.

22
23 Desirably the nucleotide binding portion is a DNA
24 binding domain of an [oestrogen] estrogen or
25 progesterone receptor, or a functional equivalent
26 thereof. Examples of sequences encoding such
27 nucleotide binding portions are set out in SEQ ID Nos
28 11 and 13.

29
30 Suitable genetic constructs according to the invention
31 include pDM12, pDM14 and pDM16, deposited at NCIMB on

1 28 August 1998 under Nos NCIMB 40970, NCIMB 40971 and
2 NCIMB 40972 respectively.

3

4 It is envisaged that a conventionally produced genetic
5 library may be exposed to the genetic construct(s)
6 described above. Thus, each individual member of the
7 genetic library will be separately incorporated into
8 the genetic construct and the library will be present
9 in the form of a library of recombinant polynucleotides
10 (as described above), usually in the form of vectors,
11 each recombinant polynucleotide including as library
12 member.

13

14 Thus, in a further aspect, the present invention
15 provides a library of recombinant polynucleotides (as
16 defined above) wherein each polynucleotide includes a
17 polynucleotide obtained from a genetic library and
18 which encodes the target peptide portion of the
19 chimeric protein expressed by the recombinant
20 polynucleotide.

21

22 Optionally, the chimeric protein may further include a
23 linker sequence located between the nucleotide binding
24 portion and the target peptide portion. The linker
25 sequence will reduce steric interference between the
26 two portions of the protein. Desirably the linker
27 sequence exhibits a degree of flexibility.

28

29 Also disclosed are methods for constructing and
30 screening libraries of PDCP particles, displaying many
31 different peptides, allowing the isolation and

1 identification of particular peptides by means of
2 affinity techniques relying on the binding activity of
3 the peptide of interest. The resulting polynucleotide
4 sequences can therefore be more readily identified, re-
5 cloned and expressed.

6

7 A method of constructing a genetic library, said method
8 comprising:

9

- 10 a) constructing multiple copies of a recombinant
11 vector comprising a polynucleotide sequence which
12 encodes a nucleotide binding portion able to
13 recognise and bind to a specific sequence motif
14 (and optionally also including the specific
15 sequence motif);
- 16
- 17 b) operably linking each said vector to a
18 polynucleotide encoding a target polypeptide, such
19 that expression of said operably linked vector
20 results in expression of a chimeric protein
21 comprising said target peptide and said nucleotide
22 binding portions; wherein said multiple copies of
23 said operably linked vectors collectively express
24 a library of target peptide portions;
- 25
- 26 c) transforming host cells with the vectors of step
27 b);
- 28
- 29 d) culturing the host cells of step c) under
30 conditions suitable for expression of said
31 chimeric protein;

- 1
2 e) providing a recombinant polynucleotide comprising
3 the nucleotide sequence motif specifically
4 recognised by the nucleotide binding portion and
5 exposing this polynucleotide to the chimeric
6 protein of step d) to yield a polynucleotide-
7 chimeric protein complex; and
8
9 f) causing production of a non-sequence-specific
10 moiety able to bind to the non-protected portion
11 of the polynucleotide encoding the chimeric
12 protein to form a peptide display carrier package.
13

14 The present invention further provides a method of
15 screening a genetic library, said method comprising:
16

- 17 a) exposing the polynucleotide members of said
18 library to multiple copies of a genetic construct
19 comprising a nucleotide sequence encoding a
20 nucleotide binding portion able to recognise and
21 bind to a specific sequence motif, under
22 conditions suitable for the polynucleotides of
23 said library each to be individually ligated into
24 one copy of said genetic construct, to create a
25 library of recombinant polynucleotides;
26
27 b) exposing said recombinant polynucleotides to a
28 population of host cells, under conditions
29 suitable for transformation of said host cells by
30 said recombinant polynucleotides;
31

- 1 c) selecting for transformed host cells;
2
3 d) exposing said transformed host cells to conditions
4 suitable for expression of said recombinant
5 polynucleotide to yield a chimeric protein; and
6
7 e) providing a recombinant polynucleotide comprising
8 the nucleotide sequence motif specifically
9 recognised by the nucleotide binding portion and
10 exposing this polynucleotide to the chimeric
11 protein of step d) to yield a polynucleotide-
12 chimeric protein complex;
13
14 f) protecting any exposed portions of the
15 polynucleotide in the complex of step e) to form a
16 peptide display carrier package; and
17
18 g) screening said peptide display carrier package to
19 select only those packages displaying a target
20 peptide portion having the characteristics
21 required.

22
23 Desirably in step a) the genetic construct is pDM12,
24 pDM14 or pDM16.

25
26 Desirably in step f) the peptide display package
27 carrier is extruded from the transformed host cell
28 without lysis of the host cell.

29
30 Generally the transformed host cells will be plated out
31 or otherwise divided into single colonies following

1 transformation and prior to expression of the chimeric
2 protein.

3
4 The screening step g) described above may look for a
5 particular target peptide either on the basis of
6 function (e.g. enzymic activity) or structure (e.g.
7 binding to a specific antibody). Once the peptide
8 display carrier package is observed to include a target
9 peptide with the desired characteristics, the
10 polynucleotide portion thereof (which of course encodes
11 the chimeric protein itself) can be amplified, cloned
12 and otherwise manipulated using standard genetic
13 engineering techniques.

14
15 The current invention differs from the prior art
16 teaching of the previous disclosures US Patent No
17 5,403,484 and US Patent No 5,571,698, as the invention
18 does not require outer surface transport signals, or
19 functional portions of viral coat proteins, to enable
20 the display of chimeric binding proteins on the outer
21 surface of the viral particle or genetic package.

22
23 The current invention also differs from the teaching of
24 WO-A-92/01047 and WO-A-92/20791, as no component of a
25 secreted replicable genetic display package, or viral
26 coat protein is required, to enable display of the
27 target peptide on the outer surface of the viral
28 particle.

29
30 The current invention differs from the teaching of US
31 Patent No 5498530, as it enables the display of

1 chimeric proteins, linked to the polynucleotide
2 encoding the chimeric protein, extra-cellularly, not in
3 the cytoplasm of a host cell. In the current invention
4 the chimeric proteins are presented on the outer
5 surface of a peptide display carrier package (PDCP)
6 which protects the DNA encoding the chimeric protein,
7 and does not require cell lysis to obtain access to the
8 chimeric protein-DNA complex. Finally, the current
9 invention does not rely upon the lacI DNA binding
10 protein to form the chimeric protein-DNA complex.

11
12 In one embodiment of the invention, the nucleotide
13 binding portion of the chimeric protein comprises a DNA
14 binding domain from one or more of the nuclear steroid
15 receptor family of proteins, or a functional equivalent
16 of such a domain. Particular examples include (but are
17 not limited to) a DNA binding domain of the [oestrogen]
18 estrogen receptor or the progesterone receptor, or
19 functional equivalents thereof. These domains can
20 recognise specific DNA sequences, termed hormone
21 response elements (HRE), which can be bound as both
22 double and single-stranded DNA. The DNA binding domain
23 of such nuclear steroid receptor proteins is preferred.

24
25 The [oestrogen] estrogen receptor is especially
26 referred to below by way of example, for convenience
27 since:

28 (a) The [oestrogen] estrogen receptor is a large
29 multifunctional polypeptide of 595 amino acids which
30 functions in the cytoplasm and nucleus of eukaryotic
31 cells (Green et al., 1986, Science 231: 1150-1154). A

1 minimal high affinity DNA binding domain (DBD) has been
2 defined between amino acids 176 and 282 (Mader et al.,
3 1993, Nucleic Acids Res. 21: 1125-1132). The
4 functioning of this domain (i.e. DNA binding) is not
5 inhibited by the presence of non-DNA binding domains at
6 both the N and C terminal ends of this domain, in the
7 full length protein.

8
9 (b) The [oestrogen] estrogen receptor DNA binding
10 domain fragment (amino acids 176-282) has been
11 expressed in *E. coli* and shown to bind to the specific
12 double stranded DNA [oestrogen] estrogen receptor
13 target HRE nucleotide sequence, as a dimer with a
14 similar affinity (0.5nM) to the parent molecule
15 (Murdoch et al. 1990, Biochemistry 29: 8377-8385; Mader
16 et al., 1993, Nucleic Acids Research 21: 1125-1132).
17 DBD dimerization on the surface of the PDCP should
18 result in two peptides displayed per particle. This
19 bivalent display can aid in the isolation of low
20 affinity peptides and peptides that are required to
21 form a bivalent conformation in order to bind to a
22 particular target, or activate a target receptor. The
23 [oestrogen] estrogen receptor is capable of binding to
24 its 38 base pair target HRE sequence, consensus
25 sequence:

- 26
27 1) 5'-TCAGGTCAGAGTGACCTGAGCTAAAATAACACATTCAG-3'
28 ("minus strand") SEQ ID No 77, and
29 2) 3'-AGTCCAGTCTCACTGGACTCGATTTTATTGTGTAAGTC-5'
30 ("plus strand") SEQ ID No 78,
31

1 with high affinity and specificity, under the salt and
2 pH conditions normally required for selection of
3 binding peptides. Moreover, binding affinity is
4 increased 60-fold for the single-stranded coding, or
5 "plus", strand (i.e. SEQ ID No 78) of the HRE
6 nucleotide sequence over the double stranded form of
7 the specific target nucleotide sequence (Peale et al.
8 1988, Proc. Natl. Acad. Sci. USA 85: 1038-1042;
9 Lannigan & Notides, 1989, Proc. Natl. Acad. Sci. USA
10 86: 863-867).

11
12 In an embodiment of the invention where the DNA binding
13 component of the peptide display carrier package is the
14 [oestrogen] estrogen receptor, the nucleotide (DNA)
15 binding portion contains a minimum sequence of amino
16 acids 176-282 of the [oestrogen] estrogen receptor
17 protein. In addition, the consensus [oestrogen]
18 estrogen receptor target HRE sequence is cloned in such
19 a way that if single stranded DNA can be produced then
20 the coding, or "plus", strand of the [oestrogen]
21 estrogen receptor HRE nucleotide sequence is
22 incorporated into single-stranded DNA. An example of a
23 vector suitable for this purpose is pUC119 (see Viera
24 et al., Methods in Enzymology, Vol 153, pages 3-11,
25 1987).

26
27 In a preferred embodiment of the invention a peptide
28 display carrier package (PDCP) can be assembled when a
29 bacterial host cell is transformed with a bacteriophage
30 vector, which vector comprises a recombinant
31 polynucleotide as described above. The expression

1 vector will also comprise the specific nucleotide motif
2 that can be bound by the nucleotide binding portion of
3 the chimeric protein. Expression of recombinant
4 polynucleotide results in the production of the
5 chimeric protein which comprises the target peptide and
6 the nucleotide binding portion. The host cell is grown
7 under conditions suitable for chimeric protein
8 expression and assembly of the bacteriophage particles,
9 and the association of the chimeric protein with the
10 specific nucleotide sequence in the expression vector.
11 In this embodiment, since the vector is a
12 bacteriophage, which replicates to produce a single-
13 stranded DNA, the nucleotide binding portion preferably
14 has an affinity for single-stranded DNA. Incorporation
15 of the vector single-stranded DNA-chimeric protein
16 complex into bacteriophage particles results in the
17 assembly of the peptide display carrier package (PDCP),
18 and display of the target peptide on the outer surface
19 of the PDCP.

20
21 In this embodiment both of the required elements for
22 producing peptide display carrier packages are
23 contained on the same vector. Incorporation of the DNA-
24 chimeric protein complex into a peptide display carrier
25 package (PDCP) is preferred as DNA degradation is
26 prevented, large numbers of PDCPs are produced per host
27 cell, and the PDCPs are easily separated from the host
28 cell without recourse to cell lysis.

29
30 In a more preferred embodiment, the vector of the is a
31 phagemid vector (for example pUC119) where expression

1 of the chimeric protein is controlled by an inducible
2 promoter. In this embodiment the PDCP can only be
3 assembled following infection of the host cell with
4 both phagemid vector and helper phage. The transfected
5 host cell is then cultivated under conditions suitable
6 for chimeric protein expression and assembly of the
7 bacteriophage particles.

8
9 In this embodiment the elements of the PDCP are
10 provided by two separate vectors. The phagemid derived
11 PDCP is superior to phagemid derived display packages
12 disclosed in WO-A-92/01047 where a proportion of
13 packages displaying bacteriophage coat protein fusion
14 proteins will contain the helper phage DNA, not the
15 fusion protein DNA sequence. In the current invention,
16 a PDCP can display the chimeric fusion protein only
17 when the package contains the specific nucleotide motif
18 recognised by the nucleotide binding portion. In most
19 embodiments this sequence will be present on the same
20 DNA segment that encodes the fusion protein. In
21 addition, the prior art acknowledges that when mutant
22 and wild type proteins are co-expressed in the same
23 bacterial cell, the wild type protein is produced
24 preferentially. Thus, when the wild type helper phage,
25 phage display system of WO-A-92/01047 is used, both
26 wild type gene pIII and target peptide-gene pIII
27 chimeric proteins are produced in the same cell. The
28 result of this is that the wild type gene pIII protein
29 is preferentially packaged into bacteriophage
30 particles, over the chimeric protein. In the current

1 invention, there is no competition with wild type
2 bacteriophage coat proteins for packaging.

3
4 Desirably the target peptide is displayed in a location
5 exposed to the external environment of the PDCP, after
6 the PDCP particle has been released from the host cell
7 without recourse to cell lysis. The target peptide is
8 then accessible for binding to its ligand. Thus, the
9 target peptide may be located at or near the N-terminus
10 or the C-terminus of a nucleotide binding domain, for
11 example the DNA binding domain of the [oestrogen]
12 estrogen receptor.

13
14 The present invention also provides a method for
15 screening a DNA library expressing one or more
16 polypeptide chains that are processed, folded and
17 assembled in the periplasmic space to achieve
18 biological activity. The PDCP may be assembled by the
19 following steps:

20
21 (a) Construction of N- or C-terminal DBD chimeric
22 protein fusions in a phagemid vector.

23 (i) When the target peptide is located at the N-
24 terminus of the nucleotide binding portion, a library
25 of DNA sequences each encoding a potential target
26 peptide is cloned into an appropriate location of an
27 expression vector (i.e. behind an appropriate promoter
28 and translation sequences and a sequence encoding a
29 signal peptide leader directing transport of the
30 downstream fusion protein to the periplasmic space) and
31 upstream of the sequence encoding the nucleotide

1 binding portion. In a preferred embodiment the DNA
2 sequence(s) of interest may be joined, by a region of
3 DNA encoding a flexible amino acid linker, to the 5'-
4 end of an [oestrogen] estrogen receptor DBD.

5 (ii) Alternatively, when the target peptide is
6 located at the C-terminus of the nucleotide binding
7 domain, a library of DNA sequences each encoding a
8 potential target peptide is cloned into the expression
9 vector so that the nucleotide sequence coding for the
10 nucleotide binding portion is upstream of the cloned
11 DNA target peptide encoding sequences, said nucleotide
12 binding portion being positioned behind an appropriate
13 promoter and translation sequences and a sequence
14 encoding a signal peptide leader directing transport of
15 the downstream fusion protein to the periplasmic space.
16 In a preferred embodiment, DNA sequence(s) of interest
17 may be joined, by a region of DNA encoding a flexible
18 amino acid linker [oestrogen] estrogen receptor DBD DNA
19 sequence.

20

21 Located on the expression vector is the specific HRE
22 nucleotide sequence recognised, and bound, by the
23 [oestrogen] estrogen receptor DBD. In order to vary the
24 number of chimeric proteins displayed on each PDCP
25 particle, this sequence can be present as one or more
26 copies in the vector.

27

28 (b) Incorporation into the PDCP. Non-lytic helper
29 bacteriophage infects host cells containing the
30 expression vector. Preferred types of bacteriophage
31 include the filamentous phage fd, fl and M13. In a

1 more preferred embodiment the bacteriophage may be
2 M13K07.

3
4 The protein(s) of interest are expressed and
5 transported to the periplasmic space, and the properly
6 assembled proteins are incorporated into the PDCP
7 particle by virtue of the high affinity interaction of
8 the DBD with the specific target nucleotide sequence
9 present on the phagemid vector DNA which is naturally
10 packaged into phage particles in a single-stranded
11 form. The high affinity interaction between the DBD
12 protein and its specific target nucleotide sequence
13 prevents displacement by bacteriophage coat proteins
14 resulting in the incorporation of the protein(s) of
15 interest onto the surface of the PDCP as it is extruded
16 from the cell.

17
18 (c) Selection of the peptide of interest. Particles
19 which display the peptide of interest are then selected
20 from the culture by affinity enrichment techniques.
21 This is accomplished by means of a ligand specific for
22 the protein of interest, such as an antigen if the
23 protein of interest is an antibody. The ligand may be
24 presented on a solid surface such as the surface of an
25 ELISA plate, or in solution. Repeating the affinity
26 selection procedure provides an enrichment of clones
27 encoding the desired sequences, which may then be
28 isolated for sequencing, further cloning and/or
29 expression.
30

1 Numerous types of libraries of peptides fused to the
2 DBD can be screened under this embodiment including:

3
4 (i) Random peptide sequences encoded by synthetic
5 DNA of variable length.

6
7 (ii) Single-chain Fv antibody fragments. These
8 consist of the antibody heavy and light chain
9 variable region domains joined by a flexible
10 linker peptide to create a single-chain antigen
11 binding molecule.

12
13 (iii) Random fragments of naturally occurring
14 proteins isolated from a cell population
15 containing an activity of interest.

16
17 In another embodiment the invention concerns methods
18 for screening a DNA library whose members require more
19 than one chain for activity, as required by, for
20 example, antibody Fab fragments for ligand binding. In
21 this embodiment heavy or light chain antibody DNA is
22 joined to a nucleotide sequence encoding a DNA binding
23 domain of, for example, the [oestrogen] estrogen
24 receptor in a phagemid vector. Typically the antibody
25 DNA library sequences for either the heavy (VH and CH1)
26 or light chain (VL and CL) genes are inserted in the 5'
27 region of the [oestrogen] estrogen receptor DBD DNA,
28 behind an appropriate promoter and translation
29 sequences and a sequence encoding a signal peptide
30 leader directing transport of the downstream fusion
31 protein to the periplasmic space.

1
2 Thus, a DBD fused to a DNA library member-encoded
3 protein is produced and assembled in to the viral
4 particle after infection with bacteriophage. The second
5 and any subsequent chain(s) are expressed separately
6 either:

7
8 (a) from the same phagemid vector containing the DBD
9 and the first polypeptide fusion protein,
10 or

11
12 (b) from a separate region of DNA which may be present
13 in the host cell nucleus, or on a plasmid, phagemid or
14 bacteriophage expression vector that can co-exist, in
15 the same host cell, with the first expression vector,
16 so as to be transported to the periplasm where they
17 assemble with the first chain that is fused to the DBD
18 protein as it exits the cell. Peptide display carrier
19 packages (PDCP) which encode the protein of interest
20 can then be selected by means of a ligand specific for
21 the protein.

22
23 In yet another embodiment, the invention concerns
24 screening libraries of bi-functional peptide display
25 carrier packages where two or more activities of
26 interest are displayed on each PDCP. In this
27 embodiment, a first DNA library sequence(s) is inserted
28 next to a first DNA binding domain (DBD) DNA sequence,
29 for example the [oestrogen] estrogen receptor DBD, in
30 an appropriate vector, behind an appropriate promoter
31 and translation sequences and a sequence encoding a

1 signal peptide leader directing transport of this first
2 chimeric protein to the periplasmic space. A second
3 chimeric protein is also produced from the same, or
4 separate, vector by inserting a second DNA library
5 sequence(s) next to a second DBD DNA sequence which is
6 different from the first DBD DNA sequence, for example
7 the progesterone receptor DBD, behind an appropriate
8 promoter and translation sequences and a sequence
9 encoding a signal peptide leader. The first, or only,
10 vector contains the specific HRE nucleotide sequences
11 for both [oestrogen] estrogen and progesterone
12 receptors. Expression of the two chimeric proteins,
13 results in a PDCP with two different chimeric proteins
14 displayed. As an example, one chimeric protein could
15 possess a binding activity for a particular ligand of
16 interest, while the second chimeric protein could
17 possess an enzymatic activity. Binding by the PDCP to
18 the ligand of the first chimeric protein could then be
19 detected by subsequent incubation with an appropriate
20 substrate for the second chimeric protein. In an
21 alternative embodiment a bi-functional PDCP may be
22 created using a single DBD, by cloning one peptide at
23 the 5'-end of the DBD, and a second peptide at the 3'-
24 end of the DBD. Expression of this single bi-functional
25 chimeric protein results in a PDCP with two different
26 activities.

27

28 We have investigated the possibility of screening
29 libraries of peptides, fused to a DNA binding domain
30 and displayed on the surface of a display package, for
31 particular peptides with a biological activity of

1 interest and recovering the DNA encoding that activity.
2 Surprisingly, by manipulating the [oestrogen] estrogen
3 receptor DNA binding domain in conjunction with M13
4 bacteriophage we have been able to construct novel
5 particles which display large biologically functional
6 molecules, that allows enrichment of particles with the
7 desired specificity.

8
9 The invention described herein provides a significant
10 breakthrough in DNA library screening technology.

11
12 The invention will now be further described by
13 reference to the non-limiting examples and figures
14 below.

15 16 **Description of Figures**

17
18 Figure 1 shows the pDM12 N-terminal fusion [oestrogen]
19 estrogen receptor DNA binding domain expression vector
20 nucleotide sequence (SEQ ID No 1), between the HindIII
21 and EcoRI restriction sites, comprising a pelB leader
22 secretion sequence (in italics) (SEQ ID No 2), multiple
23 cloning site containing SfiI and NotI sites, flexible
24 (glycine)₄-serine linker sequence (boxed), a fragment of
25 the [oestrogen] estrogen receptor gene comprising amino
26 acids 176-282 (SEQ ID No 3) of the full length
27 molecule, and the 38 base pair consensus [oestrogen]
28 estrogen receptor DNA binding domain HRE sequence.

29
30 Figure 2 shows the OD_{450nm} ELISA data for negative
31 control M13K07 phage, and single-clone PDCP display

1 culture supernatants (#1-4, see Example 3) isolated by
2 selection of the lymphocyte cDNA-pDM12 library against
3 anti-human immunoglobulin kappa antibody.

4
5 Figure 3 shows partial DNA (SEQ ID No 4) and amino acid
6 (SEQ ID No 5) sequence for the human immunoglobulin
7 kappa constant region (Kabat, E. A. et al., Sequences
8 of Proteins of Immunological Interest. 4th edition. U.S.
9 Department of Health and Human Services. 1987), and
10 ELISA positive clones #2 (SEQ ID Nos 6 and 7) and #3
11 (SEQ ID Nos 8 and 9) from Figure 2 which confirms the
12 presence of human kappa constant region DNA in-frame
13 with the pelB leader sequence (pelB leader sequence is
14 underlined, the leader sequence cleavage site is
15 indicated by an arrow). The differences in the 5'-end
16 sequence demonstrates that these two clones were
17 selected independently from the library stock. The PCR
18 primer sequence is indicated in bold, clone #2 was
19 originally amplified with CDNAPCRBAK1 and clone #3 was
20 amplified with CDNAPCRBAK2.

21
22 Figure 4 shows the pDM14 N-terminal fusion [oestrogen]
23 estrogen receptor DNA binding domain expression vector
24 nucleotide sequence (SEQ ID No 10), between the HindIII
25 and EcoRI restriction sites, comprising a pelB leader
26 secretion sequence (in italics) (SEQ ID No 11), multiple
27 cloning site containing SfiI and NotI sites, flexible
28 (glycine)₄-serine linker sequence (boxed), a fragment of
29 the [oestrogen] estrogen receptor gene comprising amino
30 acids 176-282 (see SEQ ID No 12) of the full length
31 molecule, and the two 38 base pair [oestrogen] estrogen

1 receptor DNA binding domain HRE sequences (HRE 1 and
2 HRE 2).

3
4 Figure 5 shows the pDM16 C-terminal fusion [oestrogen]
5 estrogen receptor DNA binding domain expression vector
6 nucleotide sequence (SEQ ID No 13), between the HindIII
7 and EcoRI restriction sites, comprising a pelB leader
8 secretion sequence (in italics), a fragment of the
9 [oestrogen] estrogen receptor gene comprising amino
10 acids 176-282 (SEQ ID No 14) of the full length
11 molecule, flexible (glycine)₄-serine linker sequence
12 (boxed), multiple cloning site containing SfiI and NotI
13 sites and the 38 base pair [oestrogen] estrogen
14 receptor DNA binding domain HRE sequence.

15
16 Figure 6 shows the OD_{450nm} ELISA data for N-cadherin-
17 pDM16 C-terminal display PDCP binding to anti-pan-
18 cadherin monoclonal antibody in serial dilution ELISA
19 as ampicillin resistance units (a.r.u.). Background
20 binding of negative control M13K07 helper phage is also
21 shown.

22
23 Figure 7 shows the OD_{450nm} ELISA data for *in vivo*
24 biotinylated PCC-pDM16 C-terminal display PDCP binding
25 to streptavidin in serial dilution ELISA as ampicillin
26 resistance units (a.r.u.). Background binding of
27 negative control M13K07 helper phage is also shown.

28
29 Figure 8 shows the OD_{450nm} ELISA data for a human scFv
30 PDCP isolated from a human scFv PDCP display library
31 selected against substance P. The PDCP was tested

1 against streptavidin (1), streptavidin-biotinylated
2 substance P (2), and streptavidin-biotinylated CGRP
3 (3), in the presence (B) or absence (A) of free
4 substance P.

5
6 Figure 9 shows the DNA (SEQ ID Nos 15 and 17) and amino
7 acid (SEQ ID No 16 and 18) sequence of the substance P
8 binding scFv isolated from a human scFv PDCP display
9 library selected against substance P. Heavy chain (SEQ
10 ID Nos 15 and 16) and light chain (SEQ ID Nos 17 and
11 18) variable region sequence is shown with the CDRs
12 underlined and highlighted in bold.

13 14 **Materials and Methods**

15 The following procedures used by the present applicant
16 are described in Sambrook, J., et al., 1989 supra.:
17 restriction enzyme digestion, ligation, preparation of
18 electrocompetent cells, electroporation, analysis of
19 restriction enzyme digestion products on agarose gels,
20 DNA purification using phenol/chloroform, preparation
21 of 2xTY medium and plates, preparation of ampicillin,
22 kanamycin, IPTG (Isopropyl β -D-Thiogalactopyranoside)
23 stock solutions, and preparation of phosphate buffered
24 saline.

25
26 Restriction enzymes, T4 DNA ligase and cDNA synthesis
27 reagents (Superscript plasmid cDNA synthesis kit) were
28 purchased from Life Technologies Ltd (Paisley,
29 Scotland, U.K.). Oligonucleotides were obtained from
30 Cruachem Ltd (Glasgow, Scotland, U.K.), or Genosys
31 Biotechnologies Ltd (Cambridge, U.K.). Taq DNA

1 polymerase, Wizard SV plasmid DNA isolation kits,
2 streptavidin coated magnetic beads and mRNA isolation
3 reagents (PolyAtract 1000) were obtained from Promega
4 Ltd (Southampton, Hampshire, U.K.). Taqplus DNA
5 polymerase was obtained from Stratagene Ltd (Cambridge,
6 U.K.). PBS, BSA, streptavidin, substance P and anti-pan
7 cadherin antibody were obtained from SIGMA Ltd (Poole,
8 Dorset, U.K.). Anti-M13-HRP conjugated antibody,
9 Kanamycin resistant M13K07 helper bacteriophage and
10 RNAGuard were obtained from Pharmacia Ltd (St. Albans,
11 Herts, U.K.) and anti-human Igk antibody from Harlan-
12 Seralab (Loughborough, Leicestershire, U.K.)
13 Biotinylated substance P and biotinylated calcitonin
14 gene related peptide (CGRP) were obtained from
15 Peninsula Laboratories (St. Helens, Merseyside, U.K.).
16
17 Specific embodiments of the invention are given below
18 in Examples 1 to 9.
19

1 **Example 1. Construction of a N-terminal PDCP display**
2 **phagemid vector pDM12.**

3
4 The pDM12 vector was constructed by inserting an
5 [oestrogen] estrogen receptor DNA binding domain,
6 modified by appropriate PCR primers, into a phagemid
7 vector pDM6. The pDM6 vector is based on the pUC119
8 derived phage display vector pHEN1 (Hoogenboom et al.,
9 1991, Nucleic Acids Res. 19: 4133-4137). It contains
10 (Gly)₄Ser linker, Factor Xa cleavage site, a full length
11 gene III, and streptavidin tag peptide sequence
12 (Schmidt, T.G. and Skerra, A., 1993, Protein
13 Engineering 6: 109-122), all of which can be removed by
14 NotI-EcoRI digestion and agarose gel electrophoresis,
15 leaving a pelB leader sequence, SfiI, NcoI and PstI
16 restriction sites upstream of the digested NotI site.
17 The cloned DNA binding domain is under the control of
18 the lac promoter found in pUC119.

19
20 **Preparation of pDM6**

21
22 The pDM12 vector was constructed by inserting an
23 [oestrogen] estrogen receptor DNA binding domain,
24 modified by appropriate PCR primers, into a phagemid
25 vector pDM6. The pDM6 vector is based on the gene pIII
26 phage display vector pHEN1 (Hoogenboom et al., 1991,
27 Nucleic Acids Res. 19: 4133-4137), itself derived from
28 pUC119 (Viera, J. and Messing, J., 1987, Methods in
29 Enzymol. 153: 3-11). It was constructed by amplifying
30 the pIII gene in pHEN1 with two oligonucleotides:

31

1 PDM6BAK: 5 -TTT TCT GCA GTA ATA GGC GGC CGC AGG GGG AGG
2 AGG GTC CAT CGA AGG TCG CGA AGC AGA GAC TGT TGA AAG T-3
3 (SEQ ID No 19) and

4
5 PDM6FOR: 5 - TTT TGA ATT CTT ATT AAC CAC CGA ACT GCG
6 GGT GAC GCC AAG CGC TTG CGG CCG TTA AGA CTC CTT ATT ACG
7 CAG-3 (SEQ ID No 20).

8
9 and cloning the PstI-EcoRI digested PCR product back
10 into similarly digested pHEN1, thereby removing the
11 c-myc tag sequence and supE TAG codon from pHEN1. The
12 pDM6 vector contains a (Gly)₄Ser linker, Factor Xa
13 cleavage site, a full length gene III, and streptavidin
14 tag peptide sequence (Schmidt, T.G. and Skerra, A.,
15 1993, Protein Engineering 6: 109-122), all of which can
16 be removed by NotI-EcoRI digestion and agarose gel
17 electrophoresis, leaving a pelB leader sequence, SfiI,
18 NcoI and PstI restriction sites upstream of the
19 digested NotI site. The cloned DNA binding domain is
20 under the control of the lac promoter found in pUC119.

21
22 The [oestrogen] estrogen receptor DNA binding domain
23 was isolated from cDNA prepared from human bone marrow
24 (Clontech, Palo Alto, California, U.S.A.). cDNA can be
25 prepared by many procedures well known to those skilled
26 in the art. As an example, the following method using a
27 Superscript plasmid cDNA synthesis kit can be used:

28
29 **(a) First strand synthesis.**

30

1 5µg of bone marrow mRNA, in 5µl DEPC-treated water was
 2 thawed on ice and 2µl (50pmol) of cDNA synthesis primer
 3 (5'-AAAAGCGGCCGCACTGGCCTGAGAGA(N)₆-3') (SEQ ID No 21)
 4 was added to the mRNA and the mixture heated to 70°C for
 5 10 minutes, then snap-chilled on ice and spun briefly
 6 to collect the contents to the bottom of the tube. The
 7 following were then added to the tube:

8	1000u/ml RNAGuard	1µl
9	5x first strand buffer	4µl
10	0.1M DTT	2µl
11	10mM dNTPs	1µl
12	200u/µl SuperScript II reverse transcriptase	5µl

13 The mixture was mixed by pipetting gently and incubated
 14 at 37°C for 1 hour, then placed on ice.

15

16 **(b) Second strand synthesis.**

17

18 The following reagents were added to the first strand
 19 reaction:

20	DEPC-treated water	93µl
21	5x second strand buffer	30µl
22	10mM dNTPs	3µl
23	10u/µl <i>E. coli</i> DNA ligase	1µl
24	10u/µl <i>E. coli</i> DNA polymerase	4µl
25	2u/µl <i>E. coli</i> RNase H	1µl

26 The reaction was vortex mixed and incubated at 16°C for
 27 2 hours. 2µl (10u) of T4 DNA polymerase was added and
 28 incubation continued at 16°C for 5 minutes. The reaction
 29 was placed on ice and 10µl 0.5M EDTA added, then
 30 phenol-chloroform extracted, precipitated and vacuum
 31 dried.

1

2 **(c) Sal I adaptor ligation.**

3

4 The cDNA pellet was resuspended in 25µl DEPC-treated
5 water, and ligation set up as follows.

6	cDNA	25µl
7	5x T4 DNA ligase buffer	10µl
8	1µg/µl Sal I adapters*	10µl
9	1u/µlT4 DNA ligase	5µl

10 *Sal I adapters: TCGACCCACGCGTCCG-3' (SEQ ID No 22)

11 GGGTGCCGAGGC-5' (SEQ ID No 23)

12 The ligation was mixed gently and incubated for 16
13 hours at 16°C, then phenol-chloroform extracted,
14 precipitated and vacuum dried. The cDNA/adaptor pellet
15 was resuspended in 41µl of DEPC-treated water and
16 digested with 60 units of NotI at 37°C for 2 hours, then
17 phenol-chloroform extracted, precipitated and vacuum
18 dried. The cDNA pellet was re-dissolved in 100µl TEN
19 buffer (10mM Tris pH 7.5, 0.1mM EDTA, 25mM NaCl) and
20 size fractionated using a Sephacryl S-500 HR column to
21 remove unligated adapters and small cDNA fragments
22 (<400bp) according to the manufacturers instructions.
23 Fractions were checked by agarose gel electrophoresis
24 and fractions containing cDNA less than 400 base pairs
25 discarded, while the remaining fractions were pooled.

26

27 **(d) PCR amplification of [oestrogen] estrogen receptor**
28 **DNA binding domain.**

29

30 The [oestrogen] estrogen receptor was PCR amplified
31 from 5µl (150-250ng) of bone marrow cDNA using 25pmol

1 of each of the primers pDM12FOR (SEQ ID No 24) (5'-
2 AAAAGAATTCTGAATGTGTTATTTTAGCTCAGGTCACTCTGACCTGATTATCAAG
3 ACCCCACTTCACCCCCT) and pDM12BAK (SEQ ID No 25) (5'-
4 AAAAGCGGCCGCAGGGGAGGAGGGTCCATGGAATCTGCCAAGGAG-3') in
5 two 50µl reactions containing 0.1mM dNTPs, 2.5 units
6 Taq DNA polymerase, and 1x PCR reaction buffer (10mM
7 Tris-HCl pH 9.0, 5mM KCl, 0.01% Triton X[®]-100, 1.5mM
8 MgCl₂) (Promega Ltd, Southampton, U.K.). The pDM12FOR
9 primer anneals to the 3'-end of the DNA binding domain
10 of the [oestrogen] estrogen receptor and incorporates
11 two stop codons, the 38 base pair consensus [oestrogen]
12 estrogen receptor HRE sequence, and an EcoRI
13 restriction site. The pDM12BAK primer anneals to the
14 5'-end of the DNA binding domain of the [oestrogen]
15 estrogen receptor and incorporates the (Gly)₄Ser linker
16 and the NotI restriction site.

17

18 Reactions were overlaid with mineral oil and PCR
19 carried out on a Techne PHC-3 thermal cycler for 30
20 cycles of 94°C, 1 minute; 65°C, 1 minute; 72°C, 1
21 minute. Reaction products were electrophoresed on an
22 agarose gel, excised and products purified from the gel
23 using a Geneclean II kit according to the manufacturers
24 instructions (Bio101, La Jolla, California, U.S.A.).

25

26 **(e) Restriction digestion and ligation.**

27

28 The PCR reaction appended NotI and EcoRI restriction
29 sites, the (Gly)₄Ser linker, stop codons and the 38 base
30 pair [oestrogen] estrogen receptor target HRE
31 nucleotide sequence to the [oestrogen] estrogen

1 receptor DNA binding domain sequence (see Figure 1).
2 The DNA PCR fragment and the target pDM6 vector
3 (approximately 500ng) were NotI and EcoRI digested for
4 1 hour at 37°C, and DNA purified by agarose gel
5 electrophoresis and extraction with GeneClean II kit
6 (Bio101, La Jolla, California, U.S.A.). The [oestrogen]
7 estrogen receptor DNA binding domain cassette was
8 ligated into the NotI-EcoRI digested pDM6 vector
9 overnight at 16°C, phenol/chloroform extracted and
10 precipitated then electroporated into TG1 *E. coli*
11 (genotype: K12, (Δ lac-pro), supE, thi, hsD5/F' traD36,
12 proA⁺B⁺, LacI^q, LacZ Δ 15) and plated onto 2xTY agar
13 plates supplemented with 1% glucose and 100µg/ml
14 ampicillin. Colonies were allowed to grow overnight at
15 37°C. Individual colonies were picked into 5ml 2xTY
16 supplemented with 1% glucose and 100µg/ml ampicillin
17 and grown overnight at 37°C. Double stranded phagemid
18 DNA was isolated with a Wizard SV plasmid DNA isolation
19 kit and the sequence confirmed with a Prism dyedexy
20 cycle sequencing kit (Perkin-Elmer, Warrington,
21 Lancashire, U.K.) using M13FOR (SEQ ID No 26) (5'-
22 GTAAAACGACGGCCAGT) and M13REV (SEQ ID No 27) (5'-
23 GGATAACAATTTTCACACAGG) oligonucleotides. The pDM12 PDGP
24 display vector DNA sequence between the HindIII and
25 EcoRI restriction sites is shown in Figure 1.

26

27 **Example 2. Insertion of a random-primed human**
28 **lymphocyte cDNA into pDM12 and preparation of a master**
29 **PDGP stock.**

30

1 Libraries of peptides can be constructed by many
2 methods known to those skilled in the art. The example
3 given describes a method for constructing a peptide
4 library from randomly primed cDNA, prepared from mRNA
5 isolated from a partially purified cell population.
6
7 mRNA was isolated from approximately 10^9 human
8 peripheral blood lymphocytes using a polyAtract 1000
9 mRNA isolation kit (Promega, Southampton, UK). The cell
10 pellet was resuspended in 4ml extraction buffer (4M
11 guanidine thiocyanate, 25mM sodium citrate pH 7.1, 2%
12 β -mercaptoethanol). 8ml of pre-heated (70°C) dilution
13 buffer (6xSSC, 10mM Tris pH 7.4, 1mM EDTA, 0.25% SDS,
14 1% β -mercaptoethanol) was added to the homogenate and
15 mixed thoroughly by inversion. 10 μ l of biotinylated
16 oligo-dT (50 pmol/ μ l) was added, mixed and the mixture
17 incubated at 70°C for 5 minutes. The lymphocyte cell
18 lysate was transferred to 6x 2ml sterile tubes and spun
19 at 13,000 rpm in a microcentrifuge for ten minutes at
20 ambient temperature to produce a cleared lysate. During
21 this centrifugation, streptavidin coated magnetic beads
22 were resuspended and 6ml transferred to a sterile 50ml
23 Falcon tube, then placed in the magnetic stand in a
24 horizontal position until all the beads were captured.
25 The supernatant was carefully poured off and beads
26 resuspended in 6ml 0.5xSSC, then the capture repeated.
27 This wash was repeated 3 times, and beads resuspended
28 in a final volume of 6ml 0.5xSSC. The cleared lysate
29 was added to the washed beads, mixed by inversion and
30 incubated at ambient temperature for 2 minutes, then
31 beads captured in the magnetic stand in a horizontal

1 position. The beads were resuspended gently in 2ml
2 0.5xSSC and transferred to a sterile 2ml screwtop tube,
3 then captured again in the vertical position, and the
4 wash solution discarded. This wash was repeated twice
5 more. 1ml of DEPC-treated water was added to the beads
6 and mixed gently. The beads were again captured and the
7 eluted mRNA transferred to a sterile tube. 50µl was
8 electrophoresed to check the quality and quantity of
9 mRNA, while the remainder was precipitated with 0.1
10 volumes 3M sodium acetate and three volumes absolute
11 ethanol at -80°C overnight in 4 aliquots in sterile
12 1.5ml screwtop tubes.

13

14 Double stranded cDNA was synthesised as described in
15 Example 1 using 5µg of lymphocyte mRNA as template.
16 The cDNA was PCR amplified using oligonucleotides
17 CDNAPCRFOR (SEQ ID No 28) (5'-
18 AAAGCGGCCGCACTGGCCTGAGAGA), which anneals to the cDNA
19 synthesis oligonucleotide described in Example 1 which
20 is present at the 3'-end of all synthesised cDNA
21 molecules incorporates a NotI restriction site, and an
22 equimolar mixture of CDNAPCRBAK1, CDNAPCRBAK2 and
23 CDNAPCRBAK3.

24 CDNAPCRBAK1: (SEQ ID No 29) 5'-

25 AAAAGGCCCAGCCGGCCATGGCCCAGCCCACCACGCGTCCG,

26 CDNAPCRBAK2: (SEQ ID No 30) 5'-

27 AAAAGGCCCAGCCGGCCATGGCCCAGTCCCACCACGCGTCCG,

28 CDNAPCRBAK3: (SEQ ID No 31) 5'-

29 AAAAGGCCCAGCCGGCCATGGCCCAGTACCCACCACGCGTCCG),

30 all three of which anneal to the SalI adaptor sequence
31 found at the 5'-end of the cDNA and incorporate a SfiI

1 restriction site at the cDNA 5'-end. Ten PCR reactions
2 were carried out using 2µl of cDNA (50ng) per reaction
3 as described in Example 1 using 25 cycles of 94°C, 1
4 minute; 60°C, 1 minute; 72°C, 2 minutes. The reactions
5 were pooled and a 20µl aliquot checked by agarose gel
6 electrophoresis, the remainder was phenol/chloroform
7 extracted and ethanol precipitated and resuspended in
8 100µl sterile water. 5µg of pDM12 vector DNA and
9 lymphocyte cDNA PCR product were SfiI-NotI digested
10 phenol/chloroform extracted and small DNA fragments
11 removed by size selection on Chromaspin 1000 spin
12 columns (Clontech, Palo Alto, California, U.S.A.) by
13 centrifugation at 700g for 2 minutes at room
14 temperature. Digested pDM12 and lymphocyte cDNA were
15 ethanol precipitated and ligated together for 16 hours
16 at 16°C. The ligated DNA was precipitated and
17 electroporated in to TG1 *E. coli*. Cells were grown in
18 1ml SOC medium per cuvette used for 1 hour at 37°C, and
19 plated onto 2xTY agar plates supplemented with 1%
20 glucose and 100µg/ml ampicillin. 10^{-4} , 10^{-5} and 10^{-6}
21 dilutions of the electroporated bacteria were also
22 plated to assess library size. Colonies were allowed to
23 grow overnight at 30°C. 2×10^8 ampicillin resistant
24 colonies were recovered on the agar plates.
25 The bacteria were then scraped off the plates into 40ml
26 2xTY broth supplemented with 20% glycerol, 1% glucose
27 and 100µg/ml ampicillin. 5ml was added to a 20ml 2xTY
28 culture broth supplemented with 1% glucose and 100µg/ml
29 ampicillin and infected with 10^{11} kanamycin resistance
30 units (kru) M13K07 helper phage at 37°C for 30 minutes

1 without shaking, then for 30 minutes with shaking at
2 200rpm. Infected bacteria were transferred to 200ml
3 2xTY broth supplemented with 25µg/ml kanamycin,
4 100µg/ml ampicillin, and 20µM IPTG, then incubated
5 overnight at 37°C, shaking at 200rpm. Bacteria were
6 pelleted at 4000rpm for 20 minutes in 50ml Falcon
7 tubes, and 40ml 2.5M NaCl/20% PEG 6000 was added to
8 200ml of particle supernatant, mixed vigorously and
9 incubated on ice for 1 hour to precipitate PDCP
10 particles. Particles were pelleted at 11000rpm for 30
11 minutes in 250ml Oakridge tubes at 4°C in a Sorvall RC5B
12 centrifuge, then resuspended in 2ml PBS buffer after
13 removing all traces of PEG/NaCl with a pipette, then
14 bacterial debris removed by a 5 minute 13500rpm spin in
15 a microcentrifuge. The supernatant was filtered through
16 a 0.45µm polysulfone syringe filter and stored at -20°C.

17
18 **Example 3. Isolation of human immunoglobulin kappa**
19 **light chains by repeated rounds of selection against**
20 **anti-human kappa antibody.**

21
22 For the first round of library selection a 70x11mm NUNC
23 Maxisorp Immuntube (Life Technologies, Paisley,
24 Scotland U.K.) was coated with 2.5ml of 10µg/ml of
25 anti-human kappa antibody (Seralab, Crawley Down,
26 Sussex, U.K.) in PBS for 2 hours at 37°C. The tube was
27 rinsed three times with PBS (fill & empty) and blocked
28 with 3ml PBS/2% BSA for 2 hours at 37°C and washed as
29 before. 4×10^{12} a.r.u. of pDM12-lymphocyte cDNA PDCP
30 stock was added in 2ml 2% BSA/PBS/0.05% Tween 20, and

1 incubated for 30 minutes on a blood mixer, then for 90
2 minutes standing at ambient temperature. The tube was
3 washed ten times with PBS/0.1% Tween 20, then a further
4 ten times with PBS only. Bound particles were eluted in
5 1ml of freshly prepared 0.1M triethylamine for 10
6 minutes at ambient temperature on a blood mixer. Eluted
7 particles were transferred to 0.5ml 1M Tris pH 7.4,
8 vortex mixed briefly and transferred to ice.

9
10 Neutralised particles were added to 10ml log phase TG1
11 E coli bacteria (optical density: OD_{600nm} 0.3-0.5) and
12 incubated at 37°C without shaking for 30 minutes, then
13 with shaking at 200rpm for 30 minutes. 10^{-3} , 10^{-4} & 10^{-5}
14 dilutions of the infected culture were prepared to
15 estimate the number of particles recovered, and the
16 remainder was spun at 4000 rpm for 10 minutes, and the
17 pellet resuspended in 300µl 2xTY medium by vortex
18 mixing. Bacteria were plated onto 2xTY agar plates
19 supplemented with 1% glucose and 100µg/ml ampicillin.
20 Colonies were allowed to grow overnight at 30°C.

21
22 A PDCP stock was prepared from the bacteria recovered
23 from the first round of selection, as described in
24 Example 2 from a 100ml overnight culture. 250µl of the
25 round 1 amplified PDCP stock was then selected against
26 anti-human kappa antibody as described above with the
27 tube was washed twelve times with PBS/0.1% Tween 20,
28 then a further twelve times with PBS only.

29
30 To identify selected clones, eighty-eight individual
31 clones recovered from the second round of selection

1 were then tested by ELISA for binding to anti-human
2 kappa antibody. Individual colonies were picked into
3 100µl 2xTY supplemented with 100µg/ml ampicillin and 1%
4 glucose in 96-well plates (Costar) and incubated at 37°C
5 and shaken at 200rpm for 4 hours. 25µl of each culture
6 was transferred to a fresh 96-well plate, containing
7 25µl/well of the same medium plus 10^7 k.r.u. M13K07
8 kanamycin resistant helper phage and incubated at 37°C
9 for 30 minutes without shaking, then incubated at 37°C
10 and shaken at 200rpm for a further 30 minutes. 160µl of
11 2xTY supplemented with 100µg/ml ampicillin, 25µg/ml
12 kanamycin, and 20µM IPTG was added to each well and
13 particle amplification continued for 16 hours at 37°C
14 while shaking at 200rpm. Bacterial cultures were spun
15 in microtitre plate carriers at 2000g for 10 minutes at
16 4°C in a benchtop centrifuge to pellet bacteria and
17 culture supernatant used for ELISA.

18
19 A Dynatech Immulon 4 ELISA plate was coated with
20 200ng/well anti-human kappa antibody in 100µl /well PBS
21 for one hour at 37°C. The plate was washed 2x200µl/well
22 PBS and blocked for 1 hour at 37°C with 200µl/well 2%
23 BSA/PBS and then washed 2x200µl/well PBS. 50µl PDCP
24 culture supernatant was added to each well containing
25 50µl/well 4% BSA/PBS/0.1%Tween 20, and allowed to bind
26 for 1 hour at ambient temperature. The plate was washed
27 three times with 200µl/well PBS/0.1% Tween 20, then
28 three times with 200µl/well PBS. Bound PDCPs were
29 detected with 100µl/well, 1:5000 diluted anti-M13-HRP
30 conjugate (Pharmacia) in 2% BSA/PBS/0.05% Tween 20 for

1 1 hour at ambient temperature and the plate washed six
2 times as above. The plate was developed for 5 minutes
3 at ambient temperature with 100µl/well freshly prepared
4 TMB (3,3',5,5'-Tetramethylbenzidine) substrate buffer
5 (0.005% H₂O₂, 0.1mg/ml TMB in 24mM citric acid/52mM
6 sodium phosphate buffer pH 5.2). The reaction was
7 stopped with 100µl/well 12.5% H₂SO₄ and read at 450nm.
8 (ELISA data for binding clones is shown in Figure 2).

9
10 These clones were then sequenced with M13REV primer
11 (SEQ ID No 27) as in Example 1. The sequence of two of
12 the clones isolated is shown in Figure 3 (see SEQ ID
13 Nos 7 to 10).

14

15 **Example 4. Construction of the pDM14 N-terminal display**
16 **vector**

17

18 It would be useful to design vectors that contain a
19 second DBD binding sequence, such as a second
20 [oestrogen] estrogen receptor HRE sequence, thus
21 allowing the display of increased numbers of peptides
22 per PDCP. Peale et al. (1988, Proc. Natl. Acad. Sci.
23 USA 85: 1038-1042) describe a number of [oestrogen]
24 estrogen receptor HRE sequences. These sequences were
25 used to define an HRE sequence, which differs from that
26 cloned in pDM12, which we used to create a second N-
27 terminal display vector (pDM14).

28 The oligonucleotide: 5'-AAAAGAATTCGAGGTTACATTAACCTTGT
29 CCGGTCAGACTGACCCAAGTCGACCTGAATGTGTTATTTTAG-3' (SEQ ID
30 No 32) was synthesised and used to mutagenise pDM12 by
31 PCR with pDM12BAK oligonucleotide as described in

1 Example 1 using 100ng pDM12 vector DNA as template. The
2 resulting DNA fragment, which contained the [oestrogen]
3 estrogen receptor DBD and two HRE sequences separated
4 by a SalI restriction enzyme site, was NotI-EcoRI
5 restriction enzyme digested and cloned into NotI-EcoRI
6 digested pDM12 vector DNA as described in Example 1 to
7 create pDM14. The sequence of pDM14 between the HindIII
8 and EcoRI restriction enzyme sites was checked by DNA
9 sequencing. The final vector sequence between these two
10 sites is shown in Figure 4 (see SEQ ID Nos 11 and 12).

11

12 **Example 5. Construction of the pDM16 C-terminal display**
13 **vector**

14

15 In order to demonstrate the display of peptides fused
16 to the C-terminus of a DBD on a PDCP a suitable vector,
17 pDM16, was created.

18

19 In pDM16 the pelB leader DNA sequence is fused directly
20 to the [oestrogen] estrogen receptor DBD sequence
21 removing the multiple cloning sites and the Gly₄Ser
22 linker DNA sequence found in pDM12 and pDM14, which are
23 appended to the C-terminal end of the DBD sequence
24 upstream of the HRE DNA sequence.

25

26 To create this vector two separate PCR reactions were
27 carried out on a Techne Progene thermal cycler for 30
28 cycles of 94°C, 1 minute; 60°C, 1 minute; 72°C, 1
29 minute. Reaction products were electrophoresed on an
30 agarose gel, excised and products purified from the gel
31 using a Mermaid or Geneclean II kit, respectively,

1 according to the manufacturers instructions (Biol01, La
2 Jolla, California, U.S.A.).

3
4 In the first, the 5'-untranslated region and pelB
5 leader DNA sequence was amplified from 100ng of pDM12
6 vector DNA using 50pmol of each of the oligonucleotides
7 pelBFOR (SEQ ID No 33) (5'-CCTTGGCAGATTCCATCT
8 CGGCCATTGCCGGC-3') and M13REV (SEQ ID NO 27) (see
9 above) in a 100µl reaction containing 0.1mM dNTPs, 2.5
10 units Taqplus DNA polymerase, and 1x High Salt PCR
11 reaction buffer (20mM Tris-HCl pH 9.2, 60mM KCl, 2mM
12 MgCl₂) (Stratagene Ltd, Cambridge, U.K.).

13
14 In the second, the 3'-end of the pelB leader sequence
15 and the [oestrogen] estrogen receptor DBD was amplified
16 from 100ng of pDM12 vector DNA using 50pmol of each of
17 the oligonucleotides pelBBAK (SEQ ID No 34) (5'-CCGGCAA
18 TGGCCGAGATGGAATCTGCCAAGG-3') and pDM16FOR (SEQ ID No
19 35) (5'-TTTTGTCTGACTCAATCAGTTATGCGGCCGCCAGCTGCAGG
20 AGGGCCGGCTGGGCCGACCCTCCTCCCCAGACCCCACTTCACCCC-3') in a
21 100µl reaction containing 0.1mM dNTPs, 2.5 units
22 Taqplus DNA polymerase, and 1x High Salt PCR reaction
23 buffer (Stratagene Ltd, Cambridge, U.K.). Following gel
24 purification both products were mixed together and a
25 final round of PCR amplification carried out to link
26 the two products together as described above, in a
27 100µl reaction containing 0.1mM dNTPs, 2.5 units Taq
28 DNA polymerase, and 1x PCR reaction buffer (10mM Tris-
29 HCl pH 9.0, 5mM KCl, 0.01% Triton X[®]-100, 1.5mM MgCl₂)
30 (Promega Ltd, Southampton, U.K.).

31

1 The resulting DNA fragment, was HindIII-SalI
2 restriction enzyme digested and cloned into HindIII-
3 SalI digested pDM14 vector DNA as described in Example
4 1 to create pDM16. The sequence of pDM16 between the
5 HindIII and EcoRI restriction enzyme sites was checked
6 by DNA sequencing. The final vector sequence between
7 these two sites is shown in Figure 5 (see SEQ ID Nos 13
8 and 14).

9
10 **Example 6. Display of the C-terminal fragment of human**
11 **N-cadherin on the surface of a PDCP**

12
13 cDNA libraries of peptides can be constructed by many
14 methods known to those skilled in the art. One commonly
15 used method for constructing a peptide library uses
16 oligo dT primed cDNA, prepared from polyA⁺ mRNA. In
17 this method the first-strand synthesis is carried out
18 using an oligonucleotide which anneals to the 3'-end
19 polyA tail of the mRNA composed of T_n (where n is
20 normally between 10 and 20 bases) and a restriction
21 enzyme site such as NotI to facilitate cloning of cDNA.
22 The cDNA cloned by this method is normally composed of
23 the polyA tail, the 3'- end untranslated region and the
24 C-terminal coding region of the protein. As an example
25 of the C-terminal display of peptides on a PDCP, a
26 human cDNA isolated from a library constructed by the
27 above method was chosen.

28
29 The protein N-cadherin is a cell surface molecule
30 involved in cell-cell adhesion. The C-terminal
31 cytoplasmic domain of the human protein (Genbank

1 database accession number: M34064) is recognised by a
2 commercially available monoclonal antibody which was
3 raised against the C-terminal 23 amino acids of chicken
4 N-cadherin (SIGMA catalogue number: C-1821). The 1.4kb
5 human cDNA fragment encoding the C-terminal 99 amino
6 acids, 3'- untranslated region and polyA tail (NotI
7 site present at the 3'-end of the polyA tail) was
8 amplified from approximately 20ng pDM7-NCAD#C with
9 25pmol of each oligonucleotide M13FOR (SEQ ID No 26)
10 and CDNPCRBAK1 (SEQ ID No 29) (see above) in a 50µl
11 reaction containing 0.1mM dNTPs, 2.5 units Taqplus DNA
12 polymerase, and 1x High Salt PCR reaction buffer (20mM
13 Tris-HCl pH 9.2, 60mM KCl, 2mM MgCl₂) (Stratagene Ltd,
14 Cambridge, U.K.) on a Techne Progene thermal cycler for
15 30 cycles of 94°C, 1 minute; 60°C, 1 minute; 72°C, 1
16 minute. Following gel purification and digestion with
17 SfiI and NotI restriction enzymes, the PCR product was
18 cloned into pDM16 using an analogous protocol as
19 described in Example 1.

20

21 Clones containing inserts were identified by ELISA of
22 96 individual PDCP cultures prepared as described in
23 Example 3. A Dynatech Immulon 4 ELISA plate was coated
24 with 1:250 diluted anti-pan cadherin monoclonal
25 antibody in 100µl /well PBS overnight at 4°C. The plate
26 was washed 3x200µl/well PBS and blocked for 1 hour at
27 37°C with 200µl/well 2% Marvel non-fat milk powder/PBS
28 and then washed 2x200µl/well PBS. 50µl PDCP culture
29 supernatant was added to each well containing 50µl/well
30 4% Marvel/PBS, and allowed to bind for 1 hour at
31 ambient temperature. The plate was washed three times

1 with 200µl/well PBS/0.1% Tween 20, then three times
2 with 200µl/well PBS. Bound PDCPs were detected with
3 100µl/well, 1:5000 diluted anti-M13-HRP conjugate
4 (Pharmacia) in 2% Marvel/PBS for 1 hour at ambient
5 temperature and the plate washed six times as above.
6 The plate was developed for 15 minutes at ambient
7 temperature with 100µl/well freshly prepared TMB
8 (3,3',5,5'-Tetramethylbenzidine) substrate buffer
9 (0.005% H₂O₂, 0.1mg/ml TMB in 24mM citric acid/52mM
10 sodium phosphate buffer pH 5.2). The reaction was
11 stopped with 100µl/well 12.5% H₂SO₄ and read at 450nm.
12 The nucleotide sequence of an ELISA positive clone
13 insert and DBD junction was checked by DNA sequencing
14 using oligonucleotides M13FOR (SEQ ID No 26) (see
15 Example 1) and ORSEQBAK (SEQ ID No 36) (5'-
16 TGTTGAAACACAAGCGCCAG-3').
17
18 A fifty-fold concentrated stock of C-terminal N-
19 cadherin PDCP particles was prepared by growing the un-
20 infected TG1 clone in 1ml 2xTY culture broth
21 supplemented with 1% glucose and 100µg/ml ampicillin
22 for five hours at 37°C, shaking at 200rpm and infecting
23 with 10⁸ kanamycin resistance units (kru) M13K07 helper
24 phage at 37°C for 30 minutes without shaking, then for
25 30 minutes with shaking at 200rpm. Infected bacteria
26 were transferred to 20ml 2xTY broth supplemented with
27 25µg/ml kanamycin, 100µg/ml ampicillin, and 20µM IPTG,
28 then incubated overnight at 30°C, shaking at 200rpm.
29 Bacteria were pelleted at 4000rpm for 20 minutes in
30 50ml Falcon tubes, and 4ml 2.5M NaCl/20% PEG 6000 was

1 added to 20ml of PDCP supernatant, mixed vigorously and
2 incubated on ice for 1 hour to precipitate particles.

3
4 The particles were pelleted at 11000rpm for 30 minutes
5 in 50ml Oakridge tubes at 4°C in a Sorvall RC5B
6 centrifuge, then resuspended in PBS buffer after
7 removing all traces of PEG/NaCl with a pipette, then
8 bacterial debris removed by a 5 minute 13500rpm spin in
9 a microcentrifuge. The supernatant was filtered through
10 a 0.45µm polysulfone syringe filter. The concentrated
11 stock was two-fold serially diluted and used in ELISA
12 against plates coated with anti-pan-cadherin antibody
13 as described above (see Figure 6).

14
15 This example demonstrates the principle of C-terminal
16 display using PDCPs, that C-terminal DBD-peptide fusion
17 PDCPs can be made which can be detected in ELISA, and
18 the possibility that oligo dT primed cDNA libraries may
19 be displayed using this method.

20
21 **Example 7. Display of in vivo biotinylated C-terminal**
22 **domain of human propionyl CoA carboxylase on the**
23 **surface of a PDCP**

24
25 Example 6 shows that the C-terminal domain of human N-
26 cadherin can be expressed on the surface of a PDCP as a
27 C-terminal fusion with the DBD. Here it is shown that
28 the C-terminal domain of another human protein
29 propionyl CoA carboxylase alpha chain (Genbank
30 accession number: X14608) can similarly be displayed,
31 suggesting that this methodology may be general.

1
2 The alpha sub-unit of propionyl CoA carboxylase alpha
3 chain (PCC) contains 703 amino acids and is normally
4 biotinylated at position 669. It is demonstrated that
5 the PCC peptide displayed on the PDCP is biotinylated,
6 as has been shown to occur when the protein is
7 expressed in bacterial cells (Leon-Del-Rio & Gravel;
8 1994, J. Biol. Chem. 37, 22964-22968).

9
10 The 0.8kb human cDNA fragment of PCC alpha encoding the
11 C-terminal 95 amino acids, 3'- untranslated region and
12 polyA tail (NotI site present at the 3'-end of the
13 polyA tail) was amplified and cloned into pDM16 from
14 approximately 20ng pDM7-PCC#C with 25pmol of each
15 oligonucleotide M13FOR (SEQ ID No 26) and CDNPCRBAK1
16 (SEQ ID No 29) as described in Example 6.

17
18 Clones containing inserts were identified by ELISA as
19 described in Example 6, except that streptavidin was
20 coated onto the ELISA plate at 250ng/well, in place of
21 the anti-cadherin antibody. The nucleotide sequence of
22 an ELISA positive clone insert and DBD junction was
23 checked by DNA sequencing using oligonucleotides M13FOR
24 (SEQ ID No 26) and ORSEQBAK (SEQ ID No 36) (see above).
25 A fifty-fold concentrated stock of C-terminal PCC PDCP
26 particles was prepared and tested in ELISA against
27 streptavidin as described in Example 6 (see Figure 7).

28
29 This example shows not only that the peptide can be
30 displayed as a C-terminal fusion on a PDCP, but also
31 that *in vivo* modified peptides can be displayed.

Example 8. Construction of a human scFv PDCP display library

This example describes the generation of a human antibody library of scFvs made from an un-immunised human. The overall strategy for the PCR assembly of scFv fragments is similar to that employed by Marks, J. D. et al. 1991, J. Mol. Biol. 222: 581-597. The antibody gene oligonucleotides used to construct the library are derived from the Marke et al., paper and from sequence data extracted from the Kabat database (Kabat, E. A. et al., Sequences of Proteins of Immunological Interest. 4th edition. U.S. Department of Health and Human Services. 1987). The three linker oligonucleotides are described by Zhou et al. (1994, Nucleic Acids Res., 22: 888-889), all oligonucleotides used are detailed in Table 1.

First, mRNA was isolated from peripheral blood lymphocytes and cDNA prepared for four repertoires of antibody genes IgD, IgM, Igκ and Igλ, using four separate cDNA synthesis primers. VH genes were amplified from IgD and IgM primed cDNA, and VL genes were amplified from Igκ and Igλ primed cDNA. A portion of each set of amplified heavy chain or light chain DNA was then spliced with a separate piece of linker DNA encoding the 15 amino acids (Gly₄ Ser)₃ (Huston, J. S. et al. 1989, Gene, 77: 61). The 3'-end of the VH PCR products and the 5'-end of the VL PCR products overlap the linker sequence as a result of incorporating linker

1 sequence in the JH, V κ and V λ family primer sets (Table
2 1). Each VH-linker or linker-VL DNA product was then
3 spliced with either VH or VL DNA to produce the primary
4 scFv product in a VH-linker-VL configuration. This scFv
5 product was then amplified and cloned into pDM12 as a
6 SfiI-NotI fragment, electroporated into TG1 and a
7 concentrated PDCP stock prepared.

8

9 **mRNA isolation and cDNA synthesis.**

10 Human lymphocyte mRNA was purified as described in
11 Example 2. Separate cDNA reactions were performed with
12 IGDCDNAFOR (SEQ ID No 37), IGMCDNAFOR (SEQ ID No 38),
13 IGKCDNAFOR (SEQ ID No 39) and IGLCDNAFOR (SEQ ID No 40)
14 oligonucleotides. 50pmol of each primer was added to
15 approximately 5 μ g of mRNA in 20 μ l of nuclease free
16 water and heated to 70°C for 5 minutes and cooled
17 rapidly on ice, then made up to a final reaction volume
18 of 100 μ l containing 50mM Tris pH 8.3, 75mM KCl, 3mM
19 MgCl₂, 10mM DTT, 0.5mM dNTPs, and 2000 units of
20 Superscript II reverse transcriptase (Life
21 Technologies, Paisley, Scotland, U.K.). The reactions
22 were incubated at 37°C for two hours, then heated to
23 95°C for 5 minutes.

24

25 **Primary PCRs.**

26 For the primary PCR amplifications separate
27 amplifications were set up for each family specific
28 primer with either an equimolar mixture of the JHFOR
29 primer set (SEQ ID Nos 41 to 44) for IgM and IgD cDNA,
30 or with SCFV κ FOR (SEQ ID No 51) or SCFV λ FOR (SEQ ID No

1 52) for IgK or Igλ cDNA respectively e.g. VH1BAK and
2 JHFOR set; Vk2BAK (SEQ ID No 54) and SCFVκFOR (SEQ ID
3 No 51); Vλ3aBAK (SEQ ID No 66) and SCFVλFOR (SEQ ID No
4 52) etc. Thus, for IgM, IgD and Igκ cDNA six separate
5 reactions were set up, and seven for Igλ cDNA. A 50μl
6 reaction mixture was prepared containing 2μl cDNA,
7 25pmol of the appropriate FOR and BAK primers, 0.1mM
8 dNTPs, 2.5 units Taqplus DNA polymerase, and 1x High
9 Salt PCR reaction buffer (20mM Tris-HCl pH 9.2, 60mM
10 KCl, 2mM MgCl₂) (Stratagene Ltd, Cambridge, U.K.).
11 Reactions were amplified on a Techne Progene thermal
12 cycler for 30 cycles of 94°C, 1 minute; 60°C, 1 minute;
13 72°C, 2 minutes, followed by 10 minutes at 72°C. Fifty
14 microlitres of all 25 reaction products were
15 electrophoresed on an agarose gel, excised and products
16 purified from the gel using a Geneclean II kit
17 according to the manufacturers instructions (Bio101, La
18 Jolla, California, U.S.A.). All sets of IgD, IgM, IgK
19 or Igλ reaction products were pooled to produce VH or
20 VL DNA sets for each of the four repertoires. These
21 were then adjusted to approximately 20ng/μl.

22

23 **Preparation of linker.**

24 Linker product was prepared from eight 100μl reactions
25 containing 5ng LINKAMP3T (SEQ ID No 76) template
26 oligonucleotide, 50pmol of LINKAMP3 (SEQ ID No 74) and
27 LINKAMP5 (SEQ ID No 75) primers, 0.1mM dNTPs, 2.5 units
28 Taqplus DNA polymerase, and 1x High Salt PCR reaction
29 buffer (20mM Tris-HCl pH 9.2, 60mM KCl, 2mM MgCl₂)
30 (Stratagene Ltd, Cambridge, U.K.). Reactions were

1 amplified on a Techne Progene thermal cycler for 30
2 cycles of 94°C, 1 minute; 60°C, 1 minute; 72°C, 1
3 minute, followed by 10 minutes at 72°C. All reaction
4 product was electrophoresed on a 2% low melting point
5 agarose gel, excised and products purified from the gel
6 using a Mermaid kit according to the manufacturers
7 instructions (Bio101, La Jolla, California, U.S.A.) and
8 adjusted to 5ng/μl.

9

10 **First stage linking.**

11 Four linking reactions were prepared for each
12 repertoire using 20ng of VH or VL DNA with 5ng of
13 Linker DNA in 100μl reactions containing (for IgM or
14 IgD VH) 50pmol of LINKAMPFOR and VH1-6BAK set, or,
15 50pmol LINKAMPBAK and either SCFVκFOR (Igκ) or SCFVλFOR
16 (Igλ), 0.1mM dNTPs, 2.5 units Taq DNA polymerase, and
17 1x PCR reaction buffer (10mM Tris-HCl pH 9.0, 5mM KCl,
18 0.01% Triton X[®]-100, 1.5mM MgCl₂) (Promega Ltd,
19 Southampton, U.K.). Reactions were amplified on a
20 Techne Progene thermal cycler for 30 cycles of 94°C, 1
21 minute; 60°C, 1 minute; 72°C, 2 minutes, followed by 10
22 minutes at 72°C. Reaction products were electrophoresed
23 on an agarose gel, excised and products purified from
24 the gel using a Geneclean II kit according to the
25 manufacturers instructions (Bio101, La Jolla,
26 California, U.S.A.) and adjusted to 20ng/μl.

27

28 **Final linking and reamplification.**

29 To prepare the final scFv DNA products, five 100μl
30 reactions were performed for VH-LINKER plus VL DNA,

1 and, five 100µl reactions were performed for VH plus
2 LINKER-VL DNA for each of the four final repertoires
3 (IgM VH-VK, VH-Vλ; IgD VH-VK, VH-Vλ) as described in
4 step (d) above using 20ng of each component DNA as
5 template. Reaction products were electrophoresed on an
6 agarose gel, excised and products purified from the gel
7 using a Geneclean II kit according to the manufacturers
8 instructions (Bio101, La Jolla, California, U.S.A.) and
9 adjusted to 20ng/µl. Each of the four repertoires was
10 then re-amplified in a 100µl reaction volume containing
11 2ng of each linked product, with 50pmol VHBK1-6 (SEQ
12 ID Nos 53 to 58) and either the JKFOR (SEQ ID Nos 66 to
13 70) or JλFOR (SEQ ID Nos 71 to 73) primer sets, in the
14 presence of 0.1mM dNTPs, 2.5 units Taq DNA polymerase,
15 and 1x PCR reaction buffer (10mM Tris-HCl pH 9.0, 5mM
16 KCl, 0.01% Triton X[®]-100, 1.5mM MgCl₂) (Promega Ltd,
17 Southampton, U.K.). Thirty reactions were performed per
18 repertoire to generate enough DNA for cloning.
19 Reactions were amplified on a Techne Progene thermal
20 cycler for 25 cycles of 94°C, 1 minute; 65°C, 1 minute;
21 72°C, 2 minutes, followed by 10 minutes at 72°C.
22 Reaction products were phenol-chloroform extracted,
23 ethanol precipitated, vacuum dried and re-suspended in
24 80µl nuclease free water.

25

26 **Cloning into pDM12.**

27 Each of the four repertoires was SfiI-NotI digested,
28 and electrophoresed on an agarose gel, excised and
29 products purified from the gel using a Geneclean II kit
30 according to the manufacturers instructions (Bio101, La

1 Jolla, California, U.S.A.). Each of the four
2 repertoires was ligated overnight at 16°C in 140µl with
3 10µg of SfiI-NotI cut pDM12 prepared as in Example 2,
4 and 12 units of T4 DNA ligase (Life Technologies,
5 Paisley, Scotland, U.K.). After incubation the
6 ligations were adjusted to 200µl with nuclease free
7 water, and DNA precipitated with 1µl 20mg/ml glycogen,
8 100µl 7.5M ammonium acetate and 900µl ice-cold (-20°C)
9 absolute ethanol, vortex mixed and spun at 13,000rpm
10 for 20 minutes in a microfuge to pellet DNA. The
11 pellets were washed with 500µl ice-cold 70% ethanol by
12 centrifugation at 13,000rpm for 2 minutes, then vacuum
13 dried and re-suspended in 10µl DEPC-treated water. 1µl
14 aliquots of each repertoire was electroporated into
15 80µl *E. coli* (TG1). Cells were grown in 1ml SOC medium
16 per cuvette used for 1 hour at 37°C, and plated onto
17 2xTY agar plates supplemented with 1% glucose and
18 100µg/ml ampicillin. 10^{-4} , 10^{-5} and 10^{-6} dilutions of the
19 electroporated bacteria were also plated to assess
20 library size. Colonies were allowed to grow overnight
21 at 30°C. Cloning into SfiI-NotI digested pDM12 yielded
22 an IgM-κ/λ repertoire of 1.16×10^9 clones, and an IgD-κ/λ
23 repertoire of 1.21×10^9 clones.

24

25 **Preparation of PDCP stock.**

26 Separate PDCP stocks were prepared for each repertoire
27 library. The bacteria were then scraped off the plates
28 into 30ml 2xTY broth supplemented with 20% glycerol, 1%
29 glucose and 100µg/ml ampicillin. 3ml was added to a
30 50ml 2xTY culture broth supplemented with 1% glucose

1 and 100µg/ml ampicillin and infected with 10^{11} kanamycin
2 resistance units (kru) M13K07 helper phage at 37°C for
3 30 minutes without shaking, then for 30 minutes with
4 shaking at 200rpm. Infected bacteria were transferred
5 to 500ml 2xTY broth supplemented with 25µg/ml
6 kanamycin, 100µg/ml ampicillin, and 20µM IPTG, then
7 incubated overnight at 30°C, shaking at 200rpm. Bacteria
8 were pelleted at 4000rpm for 20 minutes in 50ml Falcon
9 tubes, and 80ml 2.5M NaCl/20% PEG 6000 was added to
10 400ml of particle supernatant, mixed vigorously and
11 incubated on ice for 1 hour to precipitate PDCP
12 particles. Particles were pelleted at 11000rpm for 30
13 minutes in 250ml Oakridge tubes at 4°C in a Sorvall RC5B
14 centrifuge, then resuspended in 40ml water and 8ml 2.5M
15 NaCl/20% PEG 6000 added to reprecipitate particles,
16 then incubated on ice for 20 minutes. Particles were
17 again pelleted at 11000rpm for 30 minutes in 50ml
18 Oakridge tubes at 4°C in a Sorvall RC5B centrifuge, then
19 resuspended in 5ml PBS buffer, after removing all
20 traces of PEG/NaCl with a pipette. Bacterial debris was
21 removed by a 5 minute 13500rpm spin in a
22 microcentrifuge. The supernatant was filtered through a
23 0.45µm polysulfone syringe filter, adjusted to 20%
24 glycerol and stored at -70°C.

25

26 **Example 9. Isolation of binding activity from a N-**
27 **terminal display PDCP library of human scFvs**

28

29 The ability to select binding activities to a target of
30 interest from a human antibody library is important due

1 to the possibility of generating therapeutic human
2 antibodies. In addition, such libraries allow the
3 isolation of antibodies to targets which cannot be used
4 for traditional methods of antibody generation due to
5 toxicity, low immunogenicity or ethical considerations.
6 In this example we demonstrate the isolation of
7 specific binding activities against a peptide antigen
8 from a PDCP library of scFvs from an un-immunised
9 human.

10

11 The generation of the library, used for the isolation
12 of binding activities in this example, is described in
13 Example 8.

14

15 Substance P is an eleven amino acid neuropeptide
16 involved in inflammatory and pain responses *in vivo*. It
17 has also been implicated in a variety of disorders such
18 as psoriasis and asthma amongst others (Misery, L.
19 1997, Br. J. Dermatol., 137: 843-850; Maggi, C. A.
20 1997, Regul. Pept. 70: 75-90; Choi, D. C. & Kwon, O.J.,
21 1998, Curr. Opin. Pulm. Med., 4: 16-24). Human
22 antibodies which neutralise this peptide may therefore
23 have some therapeutic potential. As this peptide is too
24 small to coat efficiently on a tube, as described in
25 Example 3, selection of binding activities was
26 performed in-solution, using N-terminal biotinylated
27 substance P and capturing bound PDCP particles on
28 streptavidin-coated magnetic beads.

29

30 **Enrichment for substance P binding PDCP particles.**

1 An aliquot of approximately 10^{13} a.r.u. IgM and IgD scFv
2 library stock was mixed with $1\mu\text{g}$ biotinylated substance
3 P in $800\mu\text{l}$ 4% BSA/0.1% Tween 20/PBS, and allowed to
4 bind for two hours at ambient temperature. Bound PDCPs
5 were then captured onto 1ml of BSA blocked streptavidin
6 coated magnetic beads for 10 minutes at ambient
7 temperature. The beads were captured to the side of the
8 tube with a magnet (Promega), and unbound material
9 discarded. The beads were washed eight times with 1ml
10 PBS/0.1% Tween 20/ $10\mu\text{g}/\text{ml}$ streptavidin, then two times
11 with 1ml of PBS by magnetic capture and removal of wash
12 buffer. After the final wash bound PDCPs were eluted
13 with 1ml of freshly prepared 0.1M triethylamine for 10
14 minutes, the beads were captured, and eluted particles
15 transferred to 0.5ml 1M Tris-HCl pH 7.4. Neutralised
16 particles were added to 10ml log phase TG1 *E. coli*
17 bacteria and incubated at 37°C without shaking for 30
18 minutes, then with shaking at 200rpm for 30 minutes.
19 10^{-3} , 10^{-4} & 10^{-5} dilutions of the infected culture were
20 prepared to estimate the number of particles recovered,
21 and the remainder was spun at 4000 rpm for 10 minutes,
22 and the pellet resuspended in $300\mu\text{l}$ 2xTY medium by
23 vortex mixing. Bacteria were plated onto 2xTY agar
24 plates supplemented with 1% glucose and $100\mu\text{g}/\text{ml}$
25 ampicillin. Colonies were allowed to grow overnight at
26 30°C . A 100-fold concentrated PDCP stock was prepared
27 from a 200ml amplified culture of these bacteria as
28 described above, and 0.5ml used in as second round of
29 selection with 500ng biotinylated substance P. For this

1 round 100µg/ml streptavidin was included in the wash
2 buffer.

3

4 **ELISA identification of binding clones.**

5 Binding clones were identified by ELISA of 96
6 individual PDCP cultures prepared as described in
7 Example 3 from colonies recovered after the second
8 round of selection. A Dynatech Immulon 4 ELISA plate
9 was coated with 200ng/well streptavidin in 100µl /well
10 PBS for 1 hour at 37°C. The plate was washed
11 3x200µl/well PBS and incubated with 10ng/well
12 biotinylated substance P in 100µl /well PBS for 30
13 minutes at 37°C The plate was washed 3x200µl/well PBS
14 and blocked for 1 hour at 37°C with 200µl/well 2% Marvel
15 non-fat milk powder/PBS and then washed 2x200µl/well
16 PBS. 50µl PDCP culture supernatant was added to each
17 well containing 50µl/well 4% Marvel/PBS, and allowed to
18 bind for 1 hour at ambient temperature. The plate was
19 washed three times with 200µl/well PBS/0.1% Tween 20,
20 then three times with 200µl/well PBS. Bound PDCPs were
21 detected with 100µl/well, 1:5000 diluted anti-M13-HRP
22 conjugate (Pharmacia) in 2% Marvel/PBS for 1 hour at
23 ambient temperature and the plate washed six times as
24 above. The plate was developed for 10 minutes at
25 ambient temperature with 100µl/well freshly prepared
26 TMB (3,3',5,5'-Tetramethylbenzidine) substrate buffer
27 (0.005% H₂O₂, 0.1mg/ml TMB in 24mM citric acid/52mM
28 sodium phosphate buffer pH 5.2). The reaction was
29 stopped with 100µl/well 12.5% H₂SO₄ and read at 450nm.
30 Out of 96 clones tested, 10 gave signals greater than
31 twice background (background = 0.05).

1

2 **Characterization of a binding clone.**

3 A 50-fold concentrated PDCP stock was prepared from a
4 100ml amplified culture of a single ELISA positive
5 clone as described above. 10µl per well of this stock
6 was tested in ELISA as described above for binding to
7 streptavidin, streptavidin-biotinylated-substance P and
8 streptavidin-biotinylated-CGRP (N-terminal
9 biotinylated). Binding was only observed in
10 streptavidin-biotinylated-substance P coated wells
11 indicating that binding was specific. In addition,
12 binding to streptavidin-biotinylated substance P was
13 completely inhibited by incubating the PDCP with 1µg/ml
14 free substance P (see Figure 8). The scFv VH (SEQ ID
15 Nos 15 and 16) and VL (SEQ ID Nos 17 and 18) DNA and
16 amino acid sequence was determined by DNA sequencing
17 with oligonucleotides M13REV (SEQ ID No27) and ORSEQFOR
18 (SEQ ID No 36) and is shown in Figure 9.

19

20 The results indicate that target binding activities can
21 be isolated from PDCP display libraries of human scFv
22 fragments.

23

24 **Example 10**

25 In another example the invention provides methods for
26 screening a DNA library whose members require more than
27 one chain for activity, as required by, for example,
28 antibody Fab fragments for ligand binding. To increase
29 the affinity of an antibody of known heavy and light
30 chain sequence, libraries of unknown light chains
31 co-expressed with a known heavy chain are screened for

1 higher affinity antibodies. The known heavy chain
2 antibody DNA sequence is joined to a nucleotide
3 sequence encoding [a oestrogen] an estrogen receptor
4 DNA binding domain in a phage vector which does not
5 contain the [oestrogen] estrogen receptor HRE sequence.
6 The antibody DNA sequence for the known heavy chain (VH
7 and CH1) gene is inserted in the 5' region of the
8 [oestrogen] estrogen receptor DBD DNA, behind an
9 appropriate promoter and translation sequences and a
10 sequence encoding a signal peptide leader directing
11 transport of the downstream fusion protein to the
12 periplasmic space. The library of unknown light chains
13 (VL and CL) is expressed separately from a phagemid
14 expression vector which also contains the [oestrogen]
15 estrogen receptor HRE sequence. Thus when both heavy
16 and light chains are expressed in the same host cell,
17 following infection with the phage containing the heavy
18 chain-DBD fusion, the light chain phagemid vector is
19 preferentially packaged into mature phage particles as
20 single stranded DNA, which is bound by the heavy
21 chain-DBD fusion protein during the packaging process.
22 The light chain proteins are transported to the
23 periplasm where they assemble with the heavy chain that
24 is fused to the DBD protein as it exits the cell on the
25 PDCP. In this example the DBD fusion protein and the
26 HRE DNA sequences are not encoded on the same vector,
27 the unknown peptide sequences are present on the same
28 vector as the HRE sequence. Peptide display carrier
29 packages (PDCP) which encode the protein of interest
30 can then be selected by means of a ligand specific for
31 the antibody.

Table 1 (i) Oligonucleotide primers used for human scFv library construction

cDNA synthesis primers

IgMCDNAFOR	TGGAAGAGGCACGTTCTTTCTTT
IgDCDNAFOR	CTCCTTCTTACTCTTGCTGGCGGT
IgkCDNAFOR	AGACTCTCCCCTGTTGAAGCTCTT
IgλCDNAFOR	TGAAGATTCTGTAGGGGCCACTGTCTT

JHFOR primers

JH1-2FOR	TGAACCGCCTCCACCTGAGGAGACGGTGACCAGGGTGCC
JH3FOR	TGAACCGCCTCCACCTGAAGAGACGGTGACCATTGTCCC
JH4-5FOR	TGAACCGCCTCCACCTGAGGAGACGGTGACCAGGGTTCC
JH6FOR	TGAACCGCCTCCACCTGAGGAGACGGTGACCGTGGTCCC

VH familyBAKprimers

VH1BAK	TTTTTGGCCCAGCCGGCCATGGCCCAGGTGCAGCTGGTGCAGTCTGG
VH2BAK	TTTTTGGCCCAGCCGGCCATGGCCCAGGTCAACTTAAGGGAGTCTGG
VH3BAK	TTTTTGGCCCAGCCGGCCATGGCCCAGGTGCAGCTGGTGGAGTCTGG
VH4BAK	TTTTTGGCCCAGCCGGCCATGGCCCAGGTGCAGCTGCAGGAGTCGGG
VH5BAK	TTTTTGGCCCAGCCGGCCATGGCCCAGGTGCAGCTGTTGCAGTCTGC
VH6BAK	TTTTTGGCCCAGCCGGCCATGGCCCAGGTACAGCTGCAGCAGTCAGG

Light chain FOR primers

SCFVKFOR	TTATTCGCGGCCCGCTAAACAGAGGCAGTTCCAGATTTC
SCFVλFOR	GTCACTTGC GGCCCGCTACAGTGTGGCCTTGTTGGCTTG

VK family BAK primers

VK1BAK	TCTGGCGGTGGCGGATCGGACATCCAGATGACCCAGTCTCC
VK2BAK	TCTGGCGGTGGCGGATCGGATGTTGTGATGACTCAGTCTCC
VK3BAK	TCTGGCGGTGGCGGATCGGAAATTGTGTTGACGCAGTCTCC
VK4BAK	TCTGGCGGTGGCGGATCGGACATCGTGATGACCCAGTCTCC
VK5BAK	TCTGGCGGTGGCGGATCGGAAACGACACTCACGCAGTCTCC
VK6BAK	TCTGGCGGTGGCGGATCGGAAATTGTGCTGACTCAGTCTCC

JK FOR primers

JK1FOR	TTCTCGTGCGGCCGCCTAACGTTTGATTTCCACCTTGGTCCC
JK2FOR	TTCTCGTGCGGCCGCCTAACGTTTGATCTCCAGCTTGGTCCC
JK3FOR	TTCTCGTGCGGCCGCCTAACGTTTGATATCCACTTTGGTCCC
JK4FOR	TTCTCGTGCGGCCGCCTAACGTTTGATCTCCACCTTGGTCCC
JK5FOR	TTCTCGTGCGGCCGCCTAACGTTTAATCTCCAGTCGTGTCCC

Vλ family BAK primers

Vλ1BAK	TCTGGCGGTGGCGGATCGCAGTCTGTGTTGACGCAGCCGCC
Vλ2BAK	TCTGGCGGTGGCGGATCGCAGTCTGCCCTGACTCAGCCTGC

Table 1 (ii) Oligonucleotide primers used for human scFv library construction

Vλ3aBAK	TCTGGCGGTGGCGGATCGTCTATGTGCTGACTCAGCCACC
Vλ3bBAK	TCTGGCGGTGGCGGATCGTCTTCTGAGCTGACTCAGGACCC
Vλ4BAK	TCTGGCGGTGGCGGATCGCACGTTATACTGACTCAACCGCC
Vλ5BAK	TCTGGCGGTGGCGGATCGCAGGCTGTGCTCACTCAGCCGTC
Vλ6BAK	TCTGGCGGTGGCGGATCGAATTTTATGCTGACTCAGCCCCA

Jλ primers

Jλ1FOR	TTCTCGTGCGGCCGCCTAACCTAGGACGGTGACCTTGGTCCC
Jλ2-3FOR	TTCTCGTGCGGCCGCCTAACCTAGGACGGTCAGCTTGGTCCC

Jλ4-5FOR TTCTCGTGCGGCCGCCTAACCTAAAAACGGTGAGCTGGGTCCC

Linker primers

LINKAMP3 CGATCCGCCACCGCCAGA

LINKAMP5 GTCTCCTCAGGTGGAGGC

LINKAMP3T CGATCCGCCACCGCCAGAGCCACCTCCGCCTGAACCGCCTCCACCTGAGGAGAC